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THERMOPHILIC, ACIDOPHILIC BACTERIA:
IRON, SULPHUR AND MINERAL OXIDATION

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TABLE OF CONTENTS

	<u>PAGE</u>
Title Page	i
Contents	ii
Tables	vi
Figures	vii
Acknowledgements	xiii
Declaration	xiv
Summary	xv
 PART 1 GENERAL INTRODUCTION	 1
 PART 2 MATERIALS AND METHODS	 12
2.1 Sources of samples for organism isolation	13
2.2 Enrichment culture designation	13
2.3 Organisms	13
2.4 Media	14
2.4.1 Media for growth on ferrous iron	14
2.4.2 Medium for growth on yeast extract	16
2.4.3 Media for growth on sulphur and tetrathionate	16
2.4.4 Media for growth on minerals	16
2.5 Growth conditions	18
2.5.1 Growth in shaken flasks	18
2.5.2 Growth in water-jacketed vessels	18
2.6 Enrichment culture procedures	20
2.7 Preparation of pure cultures	20
2.7.1 Iron-oxidizing bacteria	20
2.7.2 Sulphur-oxidizing bacteria	21
2.7.3 Bacteria growing on yeast extract	22
2.8 Maintenance of stock cultures	22

2.9	Assays	22
2.9.1	Ferrous iron oxidation	22
2.9.2	Sulphur oxidation	23
2.9.3	Mineral oxidation	23
2.9.4	Optical density	24
2.9.5	Cell protein	24
2.9.6	Cell carbon	25
2.10	Elemental analysis of organisms	25
2.11	Electron microscopy	25
2.12	Discontinuous polyacrylamide gel electrophoresis	26
2.12.1	Preparation of cell samples for electrophoresis	26
2.12.2	Preparation and running of gels	26
2.13	Bacterial film iron oxidation	28
2.14	Materials	31
PARTS 3-7	RESULTS	32
PART 3	MODERATELY THERMOPHILIC IRON-OXIDIZING BACTERIA	33
3.1	Introduction	34
3.2	Selection of a growth medium	37
3.3	Isolation of moderately thermophilic iron-oxidizing bacteria	37
3.4	Growth on ferrous iron	39
3.5	Heterotrophic growth on yeast extract	52
3.6	Morphology	55
3.7	Comparison of isolates by electrophoresis of cell protein lysates	61
3.8	Discussion	67

	<u>PAGE</u>
PART 4 BACTERIAL FILM CONTINUOUS IRON OXIDATION	75
4.1 Introduction	76
4.2 The effect of tubing composition on iron oxidation by <u>T. ferrooxidans</u> and moderately thermophilic bacteria	79
4.3 Comparison of continuous ferrous iron oxidation by <u>T. ferrooxidans</u> and the Evenwood moderate thermophile	80
4.4 The effect of chloride ions on the continuous oxidation of ferrous iron by <u>T. ferrooxidans</u> and the Evenwood moderate thermophile.	91
4.5 Discussion	93
 PART 5 MODERATELY THERMOPHILIC SULPHUR-OXIDIZING BACTERIA	97
5.1 Introduction	98
5.2 Selection of a medium for the isolation of organisms and the growth studies	99
5.3 Isolation of sulphur-oxidizing bacteria at 37°C and 50°C	101
5.4 Growth on sulphur	101
5.5 Morphology	104
5.6 Comparison of the isolates by electrophoresis of cell proteins	107
5.7 Discussion	107
 PART 6 EXTREMELY THERMOPHILIC IRON- AND SULPHUR-OXIDIZING ACIDOPHILES	115
6.1 Introduction	116
6.2 Isolation of extremely thermophilic bacteria	119
6.3 Growth on yeast extract	120
6.4 Growth on ferrous iron	120
6.5 Growth on sulphur	126

	<u>PAGE</u>
6.6 Morphology	129
6.7 Electrophoresis of cell proteins	129
6.8 Discussion	140
 PART 7 GROWTH OF THERMOPHILES ON MINERAL SULPHIDES	 145
7.1 Introduction	146
7.2 Moderately thermophilic bacteria: pyrite oxidation	149
7.3 Moderately thermophilic bacteria: chalcopyrite oxidation	161
7.4 Extremely thermophilic bacteria: pyrite oxidation	165
7.5 Extremely thermophilic bacteria: chalcopyrite oxidation	168
7.6 Discussion	172
 PART 8 CONCLUSIONS	 177
REFERENCES	180

LIST OF TABLES

	<u>PAGE</u>
TABLE 1 The media used for growth of bacteria on ferrous iron	14
TABLE 2 The media used for growth of bacteria on sulphur	17
TABLE 3 A comparison of substrate utilization by <u>Sulfolobus</u> strains	144
TABLE 4 Rates of iron solubilization from pyrite by moderately thermophilic bacteria	157

LIST OF FIGURES

<u>FIGURE NO.</u>		<u>PAGE</u>
1	A water-jacketed culture vessel and overhead stirrer motor used in mineral oxidation/growth experiments	19
2	Diagram of continuous bacterial film iron oxidation system	30
3	Iron oxidation by thermophile TH1 in the presence of yeast extract	38
4	The pH of the medium during growth and iron oxidation by thermophile TH1	40
5	Growth of thermophile TH1 at 50°C in the ferrous iron medium supplemented with yeast extract	41
6	Culture cell protein and iron oxidation by TH1 at 50°C	43
7	The effect of temperature on growth-associated iron oxidation by moderate thermophiles in the presence of yeast extract	44
8	The effect of temperature on autotrophic growth-associated iron oxidation by moderate thermophiles	46
9	Iron oxidation during growth at 60°C of thermophile TH1 and the Kingsbury 60°C enrichment culture	47
10	The effect of temperature and pH on iron oxidation during growth of the Evenwood isolate and of the Kingsbury 60°C enrichment culture	48
11	Iron oxidation during autotrophic growth of the Birch Coppice isolate with flask gas atmospheres of air and 5% (v/v) CO ₂ in air	50

<u>FIGURE</u>	<u>NO</u>	<u>PAGE</u>
12	The effect of thiosulphate concentration on iron oxidation by the Birch Coppice isolate	51
13	Growth of TH1 on yeast extract	53
14	Semi-logarithmic plot of culture optical density and cell protein during growth of TH1 on yeast extract	54
15	Iron oxidation by TH1 in yeast extract-supplemented ferrous iron medium: the use of iron-grown and yeast extract-grown inocula	56
16	Electronmicrographs of thermophile TH1 grown on ferrous iron	57
17	Electronmicrographs of the Birch Coppice isolate grown on ferrous iron	58
18	Electronmicrograph of <u>Thiobacillus ferrooxidans</u> grown on ferrous iron	59
19	Electronmicrograph of thermophile TH1 grown on yeast extract	60
20	Electronmicrograph of the obligately heterotrophic thermophile grown on yeast extract	62
21	Electrophoretic protein patterns of moderate thermophiles grown on iron plus yeast extract	64
22	Electrophoretic protein patterns of moderate thermophiles grown autotrophically on iron	66
23	Electrophoretic protein patterns of the Birch Coppice and Kingsbury isolates grown autotrophically and in the presence of yeast extract	69
24	The effect of tubing constituents on growth of thermophile TH1	81

<u>FIGURE NO</u>		<u>PAGE</u>
25	Iron oxidation by <u>T. ferrooxidans</u> in a continuous flow vessel.	83
26	Iron oxidation by <u>T. ferrooxidans</u> in a continuous flow vessel with an increased internal surface area	86
27	Iron oxidation by the Evenwood moderate thermophile in a continuous flow vessel	88
28	Iron oxidation by the Evenwood moderate thermophile in a continuous flow vessel with an increased internal surface area	90
29	The continuous flow vessels on completion of the experiments	92
30	The pH during growth of TH1 and <u>T. ferrooxidans</u> on sulphur plus yeast extract	100
31	Sulphate production during autotrophic growth of LM7 and LM2 on sulphur	102
32	The effect of temperature on growth of BC2, BC4, BC13, LM7 and LM2 on sulphur	103
33	Electronmicrograph of moderate thermophiles LM7 and LM2 grown autotrophically on sulphur	105
34	Electronmicrograph of the moderately thermophilic sulphur-oxidizer BC13 showing a single polar flagellum	107
35	Electrophoretic protein patterns of sulphur-oxidizing bacteria	109
36	Electrophoretic protein patterns of LM2 and LM1 grown autotrophically on ferrous iron	111

<u>FIGURE NO</u>		<u>PAGE</u>
37	The growth of <u>S. acidocaldarius</u> 98-3 at different temperatures on yeast extract	121
38	The growth of <u>S. solfataricus</u> at different temperatures on yeast extract	122
39	The effect of temperature on the growth rates of <u>S. acidocaldarius</u> 98-3 and <u>S. solfataricus</u> on yeast extract	123
40	Iron oxidation by <u>S. brierleyi</u> in serial cultures	125
41	Iron oxidation by <u>Sulfolobus</u> (Lake Myvam)	127
42	The pH of the medium during growth of <u>S. acidocaldarius</u> 98-3 and <u>Sulfolobus</u> enrichment cultures on sulphur plus yeast extract	128
43	The effect of temperature on autotrophic growth of <u>Sulfolobus</u> (Lake Myvam) during oxidation of iron, sulphur and pyrite	130
44	The effect of temperature on the rate of sulphate production from sulphur during autotrophic growth of <u>Sulfolobus</u> (Lake Myvam) and moderate thermophiles	131
45	Electronmicrograph of <u>Sulfolobus</u> (Lake Myvam) grown autotrophically on pyrite	132
46	Electrophoretic protein patterns of <u>Sulfolobus</u> strains grown on yeast extract	135
47	Electrophoretic protein patterns of <u>Sulfolobus</u> (Lake Myvam) grown on pyrite and sulphur and of <u>Sulfolobus</u> strains grown on tetrathionate	137
48	Electrophoretic protein patterns of <u>Sulfolobus</u> strains grown on pyrite	139

<u>FIGURE</u>	<u>NO</u>	<u>PAGE</u>
49	The solubilization of iron from pyrite by thermophile TH1 in a yeast extract-supplemented medium	150
50	The solubilization of iron from pyrite by thermophile TH1 in a glutathione-supplemented medium	151
51	The solubilization of iron from pyrite by thermophile TH1 in a glucose-supplemented medium	153
52	The solubilization of iron from pyrite (10 gl^{-1}) by thermophile TH1 and 50°C enrichment cultures	154
53	The solubilization of iron from pyrite (50 gl^{-1}) at 50°C by thermophile TH1 and 50°C enrichment cultures in the presence of yeast extract (0.2 gl^{-1})	155
54	The solubilization of iron from pyrite (50 gl^{-1}) by thermophile TH1 and 50°C enrichment cultures in the presence of yeast extract (0.5 gl^{-1})	158
55	The solubilization of iron from pyrite by thermophile TH1 and the Kingsbury 50°C enrichment culture: growth limitation by acidity	159
56	The effect of temperature on pyrite dissolution by the Lake Myvam 40°C enrichment culture, the Birch Coppice isolate and <u>T. ferrooxidans</u>	160
57	Copper solubilization from chalcopyrite during autotrophic growth of the moderately thermophilic Birch Coppice isolate	162

<u>FIGURE</u>	<u>NO</u>	<u>PAGE</u>
58	Copper solubilization from chalcopyrite during growth of the moderately thermophilic Birch Coppice isolate in the presence of yeast extract	163
59	Copper solubilization from chalcopyrite during autotrophic growth of <u>T. ferrooxidans</u> and the Lake Myvam 40°C enrichment culture	164
60	Copper solubilization from chalcopyrite during autotrophic growth of the Alvecote isolate	166
61	The solubilization of iron from pyrite by <u>Sulfolobus</u> (Lake Myvam) and <u>S. brierleyi</u>	167
62	The effect of temperature on the solubilization of iron from pyrite during autotrophic growth of <u>Sulfolobus</u> (Birch Coppice)	169
63	Comparison of copper solubilization from chalcopyrite by <u>Sulfolobus</u> (Lake Myvam) with cultures shaken in an orbital shaker and a water bath	170
64	The effect of temperature on copper solubilization from chalcopyrite during autotrophic growth of <u>Sulfolobus</u> (Lake Myvam)	171
65	The effect of temperature on copper solubilization from chalcopyrite during growth of <u>Sulfolobus</u> (Lake Myvam) in medium supplemented with yeast extract	173

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DECLARATION

The experimental work described in this thesis was the result of original research conducted by myself under the supervision of Dr. P. R. Norris. None of the work contained in this thesis has been used in any previous application for a degree.

SUMMARY

The aim of this study was to investigate the iron- and sulphur-oxidizing activities of thermophilic bacteria with reference to the possible use of such bacteria in the extraction of metals from mineral sulphides.

The initial characterization of a range of isolates was based on growth studies with iron and sulphur substrates and on the comparison of whole cell protein electrophoresis patterns. Three groups of bacteria were isolated and studied: moderately thermophilic iron- and mineral sulphide-oxidizing bacteria, moderately thermophilic sulphur oxidizers and extremely thermophilic Sulfolobus-like organisms. Both moderately and extremely thermophilic acidophiles were isolated from hot spring and coal pile samples. The moderately thermophilic iron-oxidizing bacteria and the extreme thermophiles which were examined were sub-divided into three and four sub-groups respectively.

In a comparative study of continuous flow iron-oxidation reactors, moderate thermophiles did not produce higher rates of ferric iron production than the mesophile T. ferrooxidans but iron oxidation was less sensitive to inhibition by chloride in a vessel containing a thermophile than in a vessel operating with the mesophile.

Iron oxidation during autotrophic growth of moderately thermophilic acidophiles and the rapid dissolution of mineral sulphides during the autotrophic growth of both the moderate and the extreme thermophiles were demonstrated, thus considerably increasing the potential industrial significance of these bacteria. The yield of soluble copper from a chalcopyrite concentrate was shown to increase with temperature from relatively low yields with the mesophile T. ferrooxidans, through moderate yields with the moderately thermophilic bacteria to almost complete mineral solubilization with the newly isolated Sulfolobus strains.

P A R T 1

GENERAL INTRODUCTION

Bacterial leaching is a term used to describe the solubilization of metals from their ores with the aid of bacteria. The process depends on the bacterial oxidation of ferrous iron and sulphur and is therefore principally concerned with sulphide minerals. There is, however, very little control over the way in which bacteria influence these reactions. The oxidation of minerals by bacteria has resulted in acid and metal pollution in mining waste drainage waters (Olem & Unz, 1980), contributed to the recovery of copper and uranium from their ores (Sheffer & Evans, 1968; MacGregor, 1969) and could perhaps be used in the desulphurization of coal (Detz & Barvinchak, 1979; Kargi & Robinson, 1982a, 1982b; Murr & Mehta, 1982; Andrews & Maczuga, 1982). The role of bacteria in mining operations has been the subject of several reviews (Brierley, 1978; Kelly et al., 1979; Tuovinen & Kelly, 1972; Lundgren & Silver, 1980; Torma, 1977).

There are four basic systems of leaching. Dump leaching involves the use of low grade ore containing a variety of ore fragment sizes. The leach solution is sprinkled on and allowed to percolate through the dump and recovered in basins. The target metal is then removed and the solution recycled back to the dump. Leaching on this scale usually occurs over a period of years which is in contrast to the second method, heap leaching, which has a leach cycle measured in months. Heap leaching occurs with crushed or uncrushed ore but of a higher grade than in dump leaching. In situ leaching involves the fracturing of an ore body and injection of a liquor which is ultimately collected in wells. This method could be more environmentally acceptable than conventional mining as it reduces surface disturbance. Vat leaching tends to use the higher grade ores which have a smaller fragment size than in the other operations. The ore is contained in large non-agitated vats through which the leach solution passes. The leaching can occur over a period measured in days and

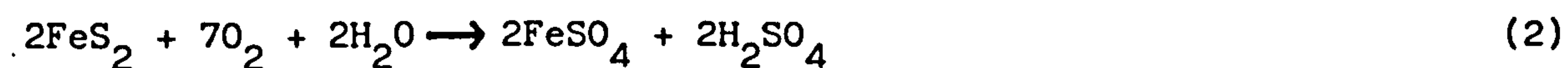
gives, in general, the highest recovery of the target metal and enables the highest degree of control over the leaching process. Using the first three methods can cause problems in ensuring an adequate air supply, in channelling of the leach liquor and with temperature variations. In all systems, heterotrophic organisms could interfere by physically blocking the sulphide surfaces and obstructing the leach solution flow (Ehrlich & Fox, 1967). The term 'vats' can also be used to describe large scale aerated, agitated fermenters containing either concentrates of a small particle size or an iron-reoxidation solution. Bacteria are used commercially in large fermenters or vats of this type for the reoxidation of iron during the production of ferric iron for the chemical extraction of uranium (Livesey-Goldblatt et al., 1977).

The bacterial leaching of metal sulphides can proceed via a direct attack on the metal sulphide or an indirect attack as a result of the bacterial production of ferric iron during oxidation. Both the direct and indirect mechanisms can operate simultaneously in mineral dissolution.

The direct attack on pyrite (equation 1) results in the production

$$4\text{FeS}_2 + 15\text{O}_2 + 2\text{H}_2\text{O} \longrightarrow 2\text{Fe}_2(\text{SO}_4)_3 + 2\text{H}_2\text{SO}_4 \quad (1)$$

of soluble ferric sulphate and sulphuric acid. Pyrite chemically degrades slowly in the presence of oxygen (equation 2) with the production of soluble ferrous sulphate.



The ferrous sulphate can then be oxidized by bacteria to produce ferric sulphate (equation 3) which will participate in the further chemical oxidation of pyrite (equation 4). The oxidation of ferrous sulphate by bacteria (equation 3) occurs many times faster than chemical oxidation (Lacey & Lawson, 1970).



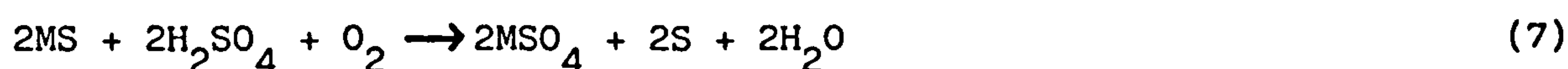
The ferrous iron produced (equation 4) can again be reoxidized by bacteria resulting in a ferric iron leaching cycle which is driven by the bacterial oxidation of ferrous iron. The sulphur produced as a result of the chemical oxidation of pyrite by ferric sulphate can itself be oxidized by bacteria to produce sulphuric acid (equation 5).



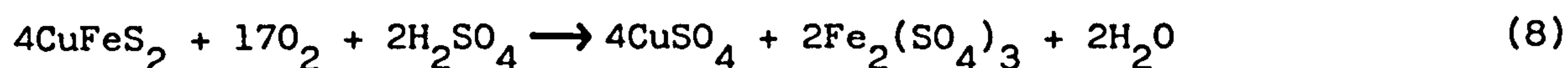
A theoretical overall equation for the direct bacterial attack on a divalent metal sulphide is



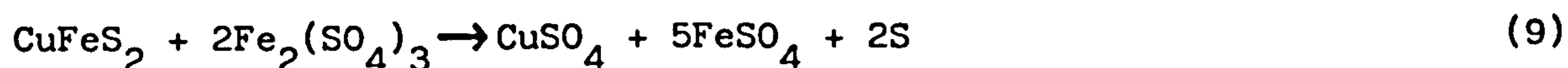
although it has been suggested (Torma et al., 1974; Tomizuka & Yagisawa, 1978) that an initial acid solubilization of metal sulphides could be important in the absence of iron with the bacteria ensuring continuous acid production (equation 7).



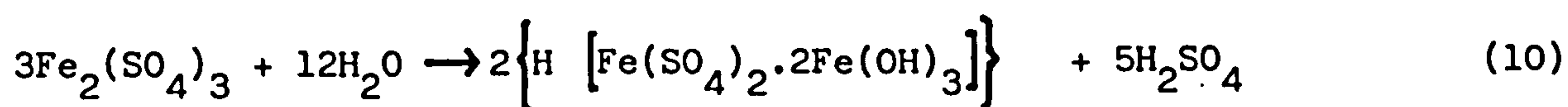
The solubilization of chalcopyrite to copper sulphate illustrates this general equation (equation 8)



However, the solubilization of chalcopyrite can also occur as a result of chemical leaching by ferric sulphate (equation 9), so a combination of 'direct' and 'indirect' bacterial attack on such sulphides is likely.



Complex, basic ferric sulphates (e.g. jarosites) can also form (equation 10) which in excess can coat the surface of minerals and so prevent any further bacterial or chemical oxidation (Guay et al., 1975; Sheffer & Evans, 1968).



Regrinding of the residue is therefore required to continue further leaching (Sakaguchi et al., 1976; McElroy & Bruynesteyn, 1978).

The great majority of work on bacterial mineral oxidation has involved the study of the chemolithoautotrophic bacterium Thiobacillus ferrooxidans (Tuovinen & Kelly, 1972; Torma, 1977). The preliminary observations of T. ferrooxidans were reported by Colmer and Hinkle (1947). Different isolates were subsequently referred to as Thiobacillus ferrooxidans (Temple & Colmer, 1951), Ferrobacillus ferrooxidans (Leathen et al., 1956) and Ferrobacillus sulfooxidans (Kinsel, 1960) until in 1972 it was decided to call them all strains of T. ferrooxidans (Kelly & Tuovinen, 1972).

T. ferrooxidans is an aerobic, acidophilic rod-shaped bacterium which is active over a pH range of 1.5 - 5.0 (Silverman, 1967) with an optimum of 2.0 - 3.5. It can however be adapted to grow at lower pH values (Tuovinen & Kelly, 1973; Kelly et al., 1977). The optimum temperature for growth is about 30°C but can vary with strain, substrate and environmental conditions (Macdonald & Clark, 1970; Silver & Torma, 1974).

T. ferrooxidans can obtain energy from the oxidation of ferrous iron, soluble and insoluble sulphides, sulphur and soluble sulphur compounds (Tuovinen & Kelly, 1972, 1973; Kelly, 1976; Kelly et al., 1977; Murr et al., 1978). It also requires a source of nitrogen with some strains demonstrating the ability to fix atmospheric nitrogen (MacKintosh, 1976, 1978) and a source of sulphate (Schnaitman et al., 1969), phosphate and trace elements.

Pure cultures of iron-grown T. ferrooxidans have been reported as having a doubling time for iron oxidation of 6.7 - 7.5 hrs at 30°C (Norris & Kelly, 1982). However, the rate of ferrous iron oxidation may be decreased in the presence of ferric iron due to the ferric iron acting as a competitive inhibitor. Variations also exist in the pyrite-oxidizing ability between cultures in terms of the length of the lag phase and the soluble iron yield (Norris & Kelly, 1982). The rates of chalcopyrite

leaching by different cultures of T. ferrooxidans can be more closely correlated to their sulphur oxidizing ability than to their iron oxidizing ability (Groudev & Genchev, 1978). Variations in chalcopyrite leaching rates can also be dependent on the previous growth substrate (Groudev & Genchev, 1978) which may be attributed to variations in copper tolerance, attachment to the mineral surface or enzyme induction between cultures. A recent comparison of 23 strains of T. ferrooxidans by DNA-DNA homology confirmed a wide genomic and physiological diversity (Harrison, 1982).

Thiobacillus thiooxidans is an acidophilic, mesophilic rod-shaped bacterium which grows on elemental sulphur and some soluble sulphur compounds but is unable to oxidize ferrous iron. Although it is unable to oxidize minerals in pure culture, it may have a role in leaching systems by oxidizing the sulphur deposited on mineral surfaces during the oxidation of sulphides by iron-oxidizing bacteria (Khalid & Ralph, 1977). It has been demonstrated to have no effect on the pyrite oxidation rate when in a mixed culture with T. ferrooxidans (Norris & Kelly, 1978).

Leptospirillum ferrooxidans is an acidophilic, vibrioid bacterium which can form long spirals when cells do not separate after cross wall formation. It was first isolated from an Armenian copper deposit (Balashova et al., 1974) and has been shown to possess a mol % GC content of 54 in comparison with 58 for T. ferrooxidans (Norris & Kelly, 1982). It is able to oxidize ferrous iron but due to its lack of sulphur-oxidizing ability (Norris & Kelly, 1982) it was initially thought to be unable to degrade minerals except in mixed culture with sulphur-oxidizing bacteria (Balashova et al., 1974; Norris & Kelly, 1978). More recently the ability of L. ferrooxidans in pure culture to cause dissolution of pyrite has been demonstrated (Norris & Kelly, 1982; Norris, 1983). It has a slower doubling time for iron oxidation at 30°C than T. ferrooxidans. A possible mechanism by which it is able to degrade pyrite without oxidizing the sulphur is via the generation of a

'ferric iron leaching cycle'. L. ferrooxidans can continue pyrite dissolution at pH values that inhibit T. ferrooxidans (Norris, 1983) but is equally sensitive to pH during the oxidation of iron. Respiratory studies have indicated that less oxygen is consumed during sulphide oxidation by L. ferrooxidans than T. ferrooxidans as might be expected due to its inability to oxidize sulphur.

The isolation of moderately and extremely thermophilic bacteria which are able to oxidize iron, sulphur and mineral sulphides, has extended the temperature range beyond that to which bacterial leaching was thought to be restricted. Temperatures of 80°C have been reported in leach dumps (Beck, 1967) so that above 45°C the thermophilic organisms might contribute to metal dissolution (Brierley & Murr, 1973; Brierley, 1974; Brierley & LeRoux, 1977) and occupy niches unsuitable for T. ferrooxidans (Brierley & Lockwood, 1977). Similarly, in hot sulphur acid soils and springs, Sulfolobus species have been isolated at temperatures above the upper limit for T. thiooxidans (Fliermans & Brock, 1972) thus providing a gradual transition of organisms in the environment whose distribution is dependent on temperature. The contribution of such organisms to the leaching environment has been reviewed (Brierley, 1978; Brock, 1978; Brierley et al., 1980; Norris & Kelly, 1982) and it has been suggested that they could provide more effective leaching of copper concentrates than at lower temperatures (Brierley & Brierley, 1978; Norris et al., 1980), accelerate pyritic oxidation during the desulphurization of coal (Detz & Barvinchak, 1979; Kargi & Robinson, 1982a, 1982b) and that the extreme thermophiles might be capable of leaching molybdenum from molybdenite which is toxic to the mesophilic bacteria (Brierley, 1974). The moderate and extremely thermophilic bacteria which oxidize iron, sulphur and mineral sulphides are described in the separate sections of this thesis.

Chemolithotrophic bacteria can obtain energy for growth and carbon assimilation from the oxidation, in the absence of light, of reduced inorganic substances (Rittenberg, 1969; Kelly, 1978; Kelly, 1981). The term autotrophic denotes organisms which fix atmospheric carbon dioxide for cell biosynthesis (Kelly, 1971; McFadden, 1973; Whittenbury & Kelly, 1977) which is in contrast to heterotrophic organisms which obtain all their carbon from organic oxidations (Whittenbury & Kelly, 1977). The term mixotrophy is used to describe the concurrent use of organic and inorganic sources of carbon and the proportion of each can vary with environmental conditions (Whittenbury & Kelly, 1977). The fixation of carbon dioxide by T. ferrooxidans via the Calvin reductive pentose phosphate cycle (Kelly, 1971; Whittenbury & Kelly, 1977; Kelly, 1978) to the level of carbohydrate is an 'energy-expensive' process and constitutes the main demand on the organism for energy. Inorganic oxidations, for example, of ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron provides a source of electrons which can be 'fed' into the electron transport system for ATP synthesis. The chemolithotrophic oxidation of iron releases relatively little energy when compared with a similar quantity of glucose (Kelly, 1978) and so organisms using this method develop a highly active oxidation mechanism which allows a rapid turnover of ferrous iron (Kelly, 1978, 1981).

There are several other factors, apart from those of temperature and pH, which can influence bacterial leaching.

The oxidation-reduction potential (Eh) is a measure of the tendency of a substance to accept or donate electrons and hence the oxidizing ability of a given environment can be determined. The oxidation by Fe^{3+} is highly dependent on this factor (Dutrizac & Macdonald, 1974).

Ammonia (nitrogen), sulphate, phosphorus, potassium and magnesium are all essential for the growth of T. ferrooxidans (Tuovinen et al.,

1971a) but the precise levels have not been clearly identified. Limiting the available oxygen can reduce both iron oxidation (Guay et al., 1975) and metal solubilization (Guay et al., 1977). Increasing the carbon dioxide concentration can increase both the rate of ferrous iron oxidation (Schnaitman & Lundgren, 1965) and metal solubilization (Torma et al., 1970; Torma et al., 1972).

The metal tolerance of T. ferrooxidans can vary between strains but the organism can tolerate aluminium (0.37M), zinc (0.15M) cobalt (0.17M), manganese (0.18M), copper (0.16M), chromium (0.1M) and uranium (0.01M) (Brierley, 1978). An increase in tolerance to uranium has been demonstrated by successive sub-culturing with increasing uranium concentrations (Tuovinen & Kelly, 1974a; 1974b). There is a lower tolerance to silver (10^{-9} M - 10^{-5} M) (Norris & Kelly, 1978a), mercury (0.03M) and the oxyanions of selenium, tellurium and arsenic are inhibitory (Brierley, 1978). The bacterial leaching of some ores might therefore be prevented or severely inhibited by the toxicity of the metal being solubilized.

The particle size affects the rate of bacterial leaching. Decreasing the particle size will lead to an increase in extraction rates (Torma, 1977; Torma & Rozgonyi, 1980) as a larger surface area will be presented for both chemical attack and bacterial attachment. The concentration of a solid substance is expressed as pulp density and at high pulp densities extraction rates can decrease due to either a lack of available nutrients (Torma, 1977) or the agitation of particulate materials, in stirred reactors, inhibiting growth of the organism (DiSpirito et al., 1981).

If an organism is to directly attack a mineral it must be in close contact with the mineral surface. Studies using the electron microscope have demonstrated the physical attachment by T. ferrooxidans, moderately

thermophilic bacteria and Sulfolobus species (Brierley & Murr, 1973; Weiss, 1973; Berry et al., 1978a; Berry & Murr, 1980) to the mineral surface. Attachment appears to be selective towards the sulphide rather than the silicate components of the matrix (Murr & Berry, 1976) and results in pitting of the surface as the sulphide mineral is oxidized (Berry et al., 1978a; Bennet & Tributsch, 1978; Hiltunen et al., 1981). The extent of the surface pitting varies between minerals. However the cytoplasm cannot come into direct contact with the mineral surface due to the cell envelope and it has been postulated (Bennett & Tributsch, 1978) that the simultaneous oxidation of iron and sulphide proceeds via secreted enzymes or other metabolites. The attachment of bacteria to the mineral surface has led to problems in enumeration which require indirect methods for measurement of bacterial growth, for example, iron oxidation, pH changes, or a measurement of metal dissolution.

It is possible that in hot regions of leach dumps, thermophiles could be useful in increasing metal extraction rates and therefore it is desirable that more is known about the type and activity of these organisms. Furthermore, the rates of bacterial concentrate leaching under controlled conditions in fermenters could be increased at higher temperatures. Therefore, the selection of the most suitable organisms for further study in pilot schemes would be an important initial step in the possible development of thermophilic, concentrate leaching. Previous studies of thermophilic mineral-oxidizing bacteria have indicated a reliance on yeast extract supplementation of the medium for good bacterial growth and activity (Brierley & Brierley, 1973; Brierley et al., 1978). The isolation and study of autotrophic thermophiles is therefore a major challenge if potentially economically advantageous thermophilic bacterial leaching is to be demonstrated.

The aim of this study, therefore, was to investigate the iron and

sulphur-oxidizing activities of thermophilic organisms and assess their significance in the field of biohydrometallurgy. Initially this was to involve obtaining, by isolation and culture donation, a broad range of moderately and extremely thermophilic bacteria for physiological comparison with T. ferrooxidans and between different cultures within the same temperature range. The most suitable organisms could then be selected for further applied work in the fields of fixed-film iron-oxidation reactors and mineral solubilization.

P A R T 2

MATERIALS AND METHODS

2.1 SOURCES OF SAMPLES FOR ORGANISM ISOLATION

Coal sediment and water samples were collected from disused coal spoil heaps at Alvecote (sample pH 3.0) and at Kingsbury (pH 3.0) in Warwickshire, U.K., and at Evenwood (sample pH 2.8) Co. Durham. Drainage water from a 2 year old, washed-coal pile was collected from the Birch Coppice Colliery, Warwickshire (sample pH 1.5, water temperature 37°C). All these samples were transferred to culture media on the day of their collection.

A hot spring water and sediment sample (pH 1.8) was obtained from Lake Myvam, Iceland and a nickel/copper mine water and sediment sample (pH 3.0) was obtained from the INCO Ltd., mines in Ontario, Canada. These samples were obtained via other persons and further information, such as the sample site temperatures, was not available. The mine water sample was transferred to culture medium within a month of collection and the hot spring sample was used to establish cultures two months after its collection.

2.2 ENRICHMENT CULTURE DESIGNATION

Enrichment cultures used in this work were named after the sample sites of their origin and after the temperatures and substrates used in their maintenance. For example, the Kingsbury 50°C pyrite enrichment culture indicates a culture maintained on pyrite at 50°C which was established after inoculation with a coal sediment and water sample from the spoil heap at Kingsbury, Warwickshire.

2.3 ORGANISMS

Culture collection strains used in this work were Sulfolobus acidocaldarius 98-3 (Deutsche Sammlung von Mikroorganismen 639), Sulfolobus solfataricus (DSM 1616),

Thiobacillus ferrooxidans (DSM 583) and Thiobacillus thiooxidans (American Type Culture Collection 8085). Sulfolobus brierleyi and a second example of Sulfolobus acidocaldarius 98-3 were provided by J.A. Brierley. Sulfolobus B6-2 (Konig et al., 1982) was provided by W. Zillig.

The moderately thermophilic iron-oxidizing bacterium isolated from an Icelandic hot spring and designated TH1 (LeRoux et al., 1977) had been studied and maintained in the Department of Environmental Sciences at Warwick University since 1977.

2.4 MEDIA

2.4.1 Media for growth on ferrous iron

The growth of TH1 at 50°C on ferrous iron was compared in the media of Leathen (Leathen et al., 1956), P. Norris (unpublished), Tuovinen and Kelly (1973) and Silverman and Lundgren (1959). The media (Table 1) were adjusted to pH 1.7 with $2\text{NH}_2\text{SO}_4$ and supplemented with yeast extract (0.2g.l^{-1}) and 50 mM ferrous iron.

The final medium selected for all experiments on growth on ferrous iron was referred to as the ferrous iron medium. The salts media were autoclaved at 15 psi/15 min.

Ferrous iron solution, made up freshly when required, containing $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (278 g.l^{-1}) and adjusted to pH 1.3 with $2\text{NH}_2\text{SO}_4$ was autoclaved at 10 psi/10 min. Unless otherwise indicated, 5 ml was added to 100 ml of sterile salts to give a final concentration of 13.9g ferrous iron per litre (50 mM). It was found that the salts media and iron solution evaporated during autoclaving and so concentrated the solutions. By using a final volume, after the ferrous iron solution had been added, of slightly over 100 mls the concentration of ferrous iron obtained

TABLE 1 The media used to examine growth of TH1 on ferrous iron

Salts (g.l ⁻¹)	Leathen	Norris	Tuovinen & Kelly	Silverman & Lundgren	Ferrous iron medium
(NH ₄) ₂ SO ₄	0.05	0.1	0.4	3.0	0.2
MgSO ₄ ·7H ₂ O	0.5	0.2	0.4	0.5	0.4
KCl	0.05	0.1	—	0.1	0.1
K ₂ HPO ₄	0.05	0.05	0.4	0.5	0.1
CaCl ₂	0.01	0.01	—	0.01	—

in the resulting medium was 50 mM.

When required yeast extract was added from a stock solution (10gl^{-1}) after autoclaving for 15 psi/15 min. Sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) was added to give the required final concentration from a stock solution (30gl^{-1}) after autoclaving at 10 psi/10 min.

2.4.2 Medium for growth on yeast extract

The ferrous iron medium (2.4.1) was supplemented with ferrous sulphate (0.01gl^{-1}) and the pH adjusted to 1.8 for growth of the moderate thermophiles and pH 2.0 for growth of the extreme thermophiles. After autoclaving at 15 psi/15 min the medium was supplemented with yeast extract to give the required concentration from a sterile stock solution (10gl^{-1}).

2.4.3 Media for growth on sulphur and tetrathionate

The growth of T. ferrooxidans and TH1 on sulphur was compared in the salts media of Bounds and Colmer (1972), Allen (1959, modified), Silverman and Lundgren (1959) and Hutchinson (Hutchinson et al., 1965) and the ferrous iron medium (2.4.1) with the addition of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01gl^{-1}) (referred to as the sulphur salts medium). The media (Table 2) were adjusted to pH 3.0 with $2\text{NH}_2\text{SO}_4$ and supplemented with sulphur flowers (5gl^{-1}).

Media were sterilized by autoclaving at 5 psi/5 min on three consecutive days and sterile yeast extract added where indicated from a sterile stock solution (10gl^{-1}).

All subsequent experiments on the growth on sulphur were carried out with the sulphur salts medium.

2.4.4 Media for growth on minerals

Experiments with 1% (w/v) mineral concentrations were carried out in the ferrous iron medium (2.4.1) adjusted to pH 2.0

TABLE 2 The media used to compare growth of T. ferrooxidans and TH1 on sulphur

Salts (gl ⁻¹)	Bounds & Colmer	Allen (modified)	Silverman & Lundgren	Hutchinson	Sulphur salts medium
(NH ₄) ₂ SO ₄	3.0	1.3	3.0	0.1	0.2
KH ₂ PO ₄	3.0	0.28	-	1.8	-
CaCl ₂ ·2H ₂ O	0.25	0.07	0.01	0.03	-
MgSO ₄ ·7H ₂ O	0.5	0.25	0.5	0.1	0.4
FeSO ₄ ·7H ₂ O	0.01	-	-	-	0.01
FeCl ₃ ·6H ₂ O		0.02	-	0.02	-
KCl			0.1	-	0.1
K ₂ HPO ₄			0.5	-	0.1
MnSO ₄ ·4H ₂ O				0.02	
NaCl				1.0	

(mg l ⁻¹)					
MnCl ₂ ·4H ₂ O		1.8			
ZnSO ₄ ·7H ₂ O		0.22			
CuCl ₂ ·2H ₂ O		0.05			
NaMoO ₄ ·2H ₂ O		0.03			
CaSO ₄		0.01			

with $2\text{NH}_2\text{SO}_4$. At higher mineral concentrations, the 9K medium of Silverman and Lundgren (1959), minus the ferrous iron and adjusted to pH 2.0, was used. Pyrite (41.8% w/w iron) and chalcopyrite (32.4% w/w copper) concentrates, ground to pass a -200 mesh sieve, were provided by N.W. LeRoux, Warren Spring Laboratory, U.K. The minerals were added to the salts media prior to autoclaving at 15 psi/15 min. When required the media were supplemented with yeast extract from a sterile stock solution. 5% (v/v) pyrite-grown inocula were used.

2.5 GROWTH CONDITIONS

2.5.1 Growth in shaken flasks

100 ml cultures were incubated in 250 ml conical flasks at 120 rpm in orbital shakers at temperatures up to 60°C. At 65°C and above flasks were incubated in water baths and shaken at a speed sufficient to keep minerals in suspension.

For autotrophic growth, 5% (v/v) CO_2 in air was supplied continuously via glass tubes inserted through foam flask bungs.

Water loss was replaced by adding sterile distilled water to maintain constant flask weights after allowing for any samples removed.

2.5.2 Growth in water-jacketed vessels

300 ml cultures were stirred in water-jacketed, glass culture vessels fitted with reflux condensers to counteract evaporation. Stirring of iron, sulphur, yeast extract and tetrathionate-grown cultures was by a magnetic stirrer bar and unit while minerals were stirred by a paddle controlled by an overhead motor (Fig. 1). Aeration was by a sparged air inlet with 50 ml min^{-1} air or 5% (v/v) CO_2 in air for autotrophic growth.



Fig. 1 A water-jacketed culture vessel (500 ml) and an overhead stirrer motor used in mineral oxidation/growth experiments.

2.6 ENRICHMENT CULTURE PROCEDURES

Enrichment cultures on ferrous iron and pyrite were established using the ferrous iron medium (2.4.1) at pH 1.7 and 2.0 respectively and containing $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.75gl^{-1}) or FeS_2 (2gl^{-1}). A low concentration of substrate was used initially and the concentration gradually increased over successive sub-cultures to reach experimental concentrations (2.4.1 and 2.4.4). The sulphur salts medium (2.4.3) was used for enrichment of sulphur-oxidizing organisms.

With samples from each sample site, half of the flasks for each substrate were supplemented with yeast extract (0.2gl^{-1}) and the other half were continuously supplied with 5% (v/v) CO_2 in air. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (0.2gl^{-1}) was added to the ferrous iron enrichment cultures.

A 3-5% (v/v) inoculum of slurry was used to establish all cultures at 50°C and 65°C . Cultures were frequently examined microscopically and serially sub-cultured into similar media when substantial growth had occurred.

Enrichment cultures at 30°C and 37°C , using the Birch Coppice and Lake Myvam samples, were established in the laboratory by P. Norris.

2.7 PREPARATION OF PURE CULTURES

2.7.1 Iron-oxidizing bacteria

Single colonies of autotrophic iron-oxidizing bacteria were obtained, after serial dilution in a modified Manning (1975) salts medium, on ferrous iron agar spread plates. Modified Manning salts containing (gl^{-1}) $(\text{NH}_4)_2\text{SO}_4$ (6.0), KCl (0.2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), $\text{Ca}(\text{NO}_3)_2$ (0.02) and K_2HPO_4 (0.01) at pH 3.0 were supplemented with $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.78gl^{-1}) and $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$

(0.2 gl^{-1}) and solidified with 0.5% (w/v) Japanese Agar. The ferrous iron, thiosulphate and agar were sterilized separately at 10 psi/10 min before being added to the warm sterile salts medium. Solidification with 0.7% (w/v) Oxoid No.1 Agar also enabled colony growth.

Plates were incubated in sealed bags in an atmosphere of 5% (v/v) CO_2 in air at the appropriate enrichment temperature.

After 5-10 days single colonies were transferred into 10 ml ferrous sulphate and thiosulphate supplemented ferrous iron medium and incubated in a 5% (v/v) CO_2 in air atmosphere. After growth had occurred the purification procedure was repeated and the resulting pure culture designated as an isolate of the sample site.

2.7.2 Sulphur-oxidizing bacteria

Single colonies of sulphur-oxidizing bacteria were obtained on thiosulphate or tetrathionate agar spread plates after serial dilution in Bounds and Colmer salts (2.4.3) at pH 3.0. Sterile $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (3gl^{-1}) or $\text{K}_2\text{S}_4\text{O}_6$ (3gl^{-1}) was added to sterile Bounds and Colmer salts, adjusted to pH 3.0 with $2\text{NH}_2\text{SO}_4$ and solidified with 0.7% (w/v) Oxoid No.1 Agar. The agar and thiosulphate or tetrathionate were mixed with the salts after separate sterilization at 10 psi/10 min.

The plates were incubated in sealed bags at the isolation temperature in an atmosphere of 5% (v/v) CO_2 in air. After 2-4 weeks single colonies were transferred to 10 ml of sulphur salts (2.4.3) and incubated in a 5% (v/v) CO_2 in air atmosphere. After purification samples were designated as isolates of the sample site.

2.7.3 Bacteria growing on yeast extract

Single colonies of heterotrophic bacteria were obtained, after serial dilution, on yeast extract medium (2.4.2) supplemented with yeast extract (0.2gl^{-1}) and solidified with 0.7% (w/v) Oxoid No.1 Agar.

2.8 MAINTENANCE OF STOCK CULTURES

Moderately thermophilic iron-oxidizing bacteria were grown on pyrite (10gl^{-1}) at 50°C in the presence of yeast extract (0.2gl^{-1}) until substantial growth had occurred. Cultures were then kept at room temperature and sub-cultured every 6 months.

Moderately thermophilic sulphur-oxidizing bacteria were grown on sulphur (5gl^{-1}) at 50°C in the presence of yeast extract (0.2gl^{-1}) and when substantial growth had occurred kept at room temperature and sub-cultured every 2 months.

The extreme thermophiles were grown on sulphur (5gl^{-1}) in the presence of yeast extract (0.2gl^{-1}) in a standing incubator at 65°C . Sub-culturing was necessary every 2 weeks. Leaving cultures at 65°C for 4 weeks or keeping cultures at room temperature or 4°C resulted in a loss of viability.

Autotrophically-grown cultures for experimental inocula were obtained from stock cultures by a minimum of 2 serial sub-cultures in the absence of yeast extract.

2.9 ASSAYS

2.9.1 Ferrous Iron Oxidation

Iron oxidation was determined by measuring the ferrous iron concentration in solution. 1 ml culture samples were placed in 2 ml of $2\text{NH}_2\text{SO}_4$ and titrated against 0.005N ceric sulphate using 1,10-phenanthroline-ferrous sulphate complex as an indicator. The decrease in ferrous iron concentration with time was

expressed in terms of percentage ferrous iron oxidized.

2.9.2 Sulphur Oxidation

Sulphur oxidation was roughly followed by measuring the drop in culture pH using a Radiometer pHM62 standard pH meter standardized with a range of buffers from pH 1.0-4.0. The pH 1.0 buffer contained 250 ml 0.2M KCl and 670 ml 0.2M HCl made up to 1 litre with distilled water.

Sulphur oxidation was measured by the rate of sulphate production. 1.5 ml culture samples were centrifuged in a micro-angle centrifuge to remove any elemental sulphur and then 1 ml of the supernatants were transferred to 9 ml of 1% HCl and stored in screw-top vials at room temperature. For analysis, equal volumes of sample (diluted when necessary with 1% HCl) and BaCl_2 (0.3557g l^{-1} ; Ba^{2+} , 0.2g l^{-1} in 1% HCl) were mixed and left to stand overnight. Residual barium was measured by atomic absorption spectrophotometry at 554.6 nm using a Rank Hilger Atomspek H1550.

A range of sulphate standards ($10\text{--}200\text{ mg l}^{-1}$) were made up fresh each time from a stock solution containing Na_2SO_4 (1.478 g l^{-1} ; SO_4 , 1 g l^{-1} in 1% HCl) and treated identically to the samples.

2.9.3 Mineral Oxidation

Solubilization of iron and copper from mineral sulphides was measured by atomic absorption spectrophotometry using a Rank Hilger Atomspek H1550. 0.35 ml culture volumes were centrifuged for 2 minutes and then 0.2 ml of the supernatants transferred into 9.8 ml of 1% HCl and stored at room temperature in screw-top vials. The levels of iron and copper in solution were read directly, or after sample dilution in 1% HCl, at 248.3 nm and

The blank contained 2 ml of distilled water and was treated in the same way as the samples. A range of standards up to 200 μg protein ml^{-1} were made from a stock solution containing 1 mg bovine albumin ml^{-1} and treated identically to the samples.

2.9.6 Cell Carbon

Samples (4-12 ml) were taken in triplicate from 1 litre shaken cultures and centrifuged for 10 minutes in a micro-angle centrifuge. The resulting pellet was washed twice with 4 ml of distilled water, adjusted to pH 2.0 with $2\text{NH}_2\text{SO}_4$, and resuspended in approximately 0.25 ml of distilled water before being stored at -18°C .

For analysis, samples were made up to 1 ml with distilled water and mixed immediately prior to injecting a 50 μl sample into a Beckman Total Organic Carbon Analyser Model 915B. A range of carbon standards ($10\text{-}100\ \mu\text{g}\ \text{ml}^{-1}$) were prepared from a stock solution of 1g anhydrous potassium biphthalate per litre.

2.10 ELEMENTAL ANALYSIS OF ORGANISMS

A cell pellet was obtained from 1 litre of cells, grown on ferrous iron in the presence of yeast extract, and washed 4 times with distilled water adjusted to pH 2.0. The pellet was dried at 105°C until a constant dry weight was reached. Analyses for carbon, nitrogen and hydrogen were carried out by the Isotope Unit, Queen Elizabeth College, University of London, using a Perkin-Elmer Elemental Analyzer.

2.11 ELECTRON MICROSCOPY

Culture specimens were fixed onto membrane-coated grids and viewed on a JEOL 1000 electron microscope.

Membranes were prepared by immersing a microscope slide in a solution of 1% (w/v) Formvar in chloroform and then allowing the membrane to dry for 5 minutes in the chloroform vapour. The

membrane was then scored and floated off onto water. Athene grids were placed matt side down onto the membrane and the coated grids removed from the water by scooping them off with a piece of stiff aluminium foil. The grids were allowed to completely dry before use.

Cells growing on ferrous iron or yeast extract were concentrated six-fold by centrifugation before being fixed on the grids. Cells growing on sulphur or pyrite were used without pre-concentration.

A single drop of culture suspension was placed on the grid and the cells fixed by placing the grid in the vapour above a 1% (w/v) solution of OsO_4 for 5 minutes. Excess liquid was carefully removed with filter paper. Where indicated in the text, cells were stained lightly (for 5 seconds) or heavily (for 2 minutes) using a 1% (w/v) uranyl acetate solution.

2.12 DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS

2.12.1 Preparation of samples for electrophoresis

Cultures in the late exponential phase of growth were centrifuged in a Beckman JR-21 centrifuge. The pellets were washed twice with distilled water adjusted to pH 2.0 with $2\text{NH}_2\text{SO}_4$ and stored at -18°C .

For analysis, cells were lysed by adding 100 μl of sample buffer containing 25% (v/v) Tris-S.D.S. stock (pH 6.8), 5% (w/v) sodium lauryl sulphate (S.D.S), 25% (v/v) glycerol, 12.5% (v/v) 2-mercaptoethanol, 0.1% (v/v) of 1% (w/v) Bromophenol Blue and 100 μl of 25% (w/v) S.D.S. to the sample and boiling for 5 minutes in a water bath. The pH of the sample was made slightly alkaline by addition of 1% (w/v) NaOH. Adjustments to the protein concentration were made, after a preliminary gel run, by adding further samples or diluting with sample buffer as required.

2.12.2 Preparation and running of gels

S.D.S.-polyacrylamide gel electrophoresis with discontinuous buffers were prepared as described by Laemmli (1969) with modifications given in

the LKB Electrophoresis Laboratory Manual.

The resolving gel was formed by combining:-

10 ml of acrylamide stock containing 30% (w/v) purified acrylamide and 0.8% (w/v) N'-N methylenebisacrylamide.

15 ml of Tris pH 8.8 buffer containing (gl^{-1}) Trizma-base (71.0),

Trizma-HCl (47.2), S.D.S. (2.0) adjusted to pH 8.8

1.5 ml of 1% (w/v) ammonium persulphate

3.5 ml of distilled water

7.5 μl of N'-N-tetramethyl-ethylenediamine (TEMED)

The gel solution was pipetted immediately between 2 glass plates, overlaid with 1 cm of distilled water and left to polymerize. After a minimum of 30 minutes, the water was poured off and the surface dried with filter paper. The stacking gel contained:-

1 ml of acrylamide stock

5 ml of Tris pH 6.8 buffer containing (gl^{-1}) Trizma-HCl (39.4)

S.D.S. (2.0) adjusted to pH 6.8

1 ml of 1% (w/v) ammonium persulphate

3 ml of distilled water

5 μl of TEMED

The complete gel was overlaid with distilled water and left overnight before running. The acrylamide stock was kept in the dark at 4°C and the ammonium persulphate solution was made up fresh each time.

Sample volumes (10-25 μl) were loaded onto the gel under running buffer containing (gl^{-1}) Trizma base (3.03), glycine (14.4) and S.D.S. (1.0) adjusted to pH 8.3; and run under a constant voltage of 120 volts with a maximum current of 40 milliamps.

On completion, gels were removed from the glass plates and the protein stained overnight in stain containing 0.05% (w/v) PAGE blue, 20% (v/v) methanol and 10% (v/v) glacial acetic acid. They were destained in a solution containing 10% (v/v) methanol and 10% (v/v)

glacial acetic acid before being photographed using back illumination.

2.13 BACTERIAL FILM IRON OXIDATION

Experiments on continuous iron oxidation by the mesophile T. ferrooxidans and a moderate thermophile from Evenwood were made in 4 water-jacketed glass vessels (Fig. 2).

Vessels 1 and 3 contained an initial volume of 330 ml and a surface area for bacterial attachment of 362 cm^2 .

Vessels 2 and 4 contained an initial volume of 220 ml and an increased surface area of $1,386 \text{ cm}^2$ made by stacking glass microscope slides in a perspex carousel.

Vessels 1 and 2 were inoculated with T. ferrooxidans and run at 30°C and vessels 3 and 4 with the Evenwood isolate and run at 50°C .

The air (80 ml min^{-1}) entered through narrow glass tubes near the magnetic stirrer bar and was hence distributed throughout the medium. Medium entered at the base of the vessels through narrow glass funnels. All connections were made of glass or PTFE with the exception of a small length of silicone tubing through the peristaltic pump. The ferrous iron medium (2.4.1) was at $\text{pH } 2.0 \pm 0.1$ for T. ferrooxidans and at $\text{pH } 1.6 \pm 0.1$ for the Evenwood isolate. The salts were supplemented with ferrous iron ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 24.9 g l^{-1}) and yeast extract (0.2 g l^{-1}) was added to the medium for the Evenwood isolate.

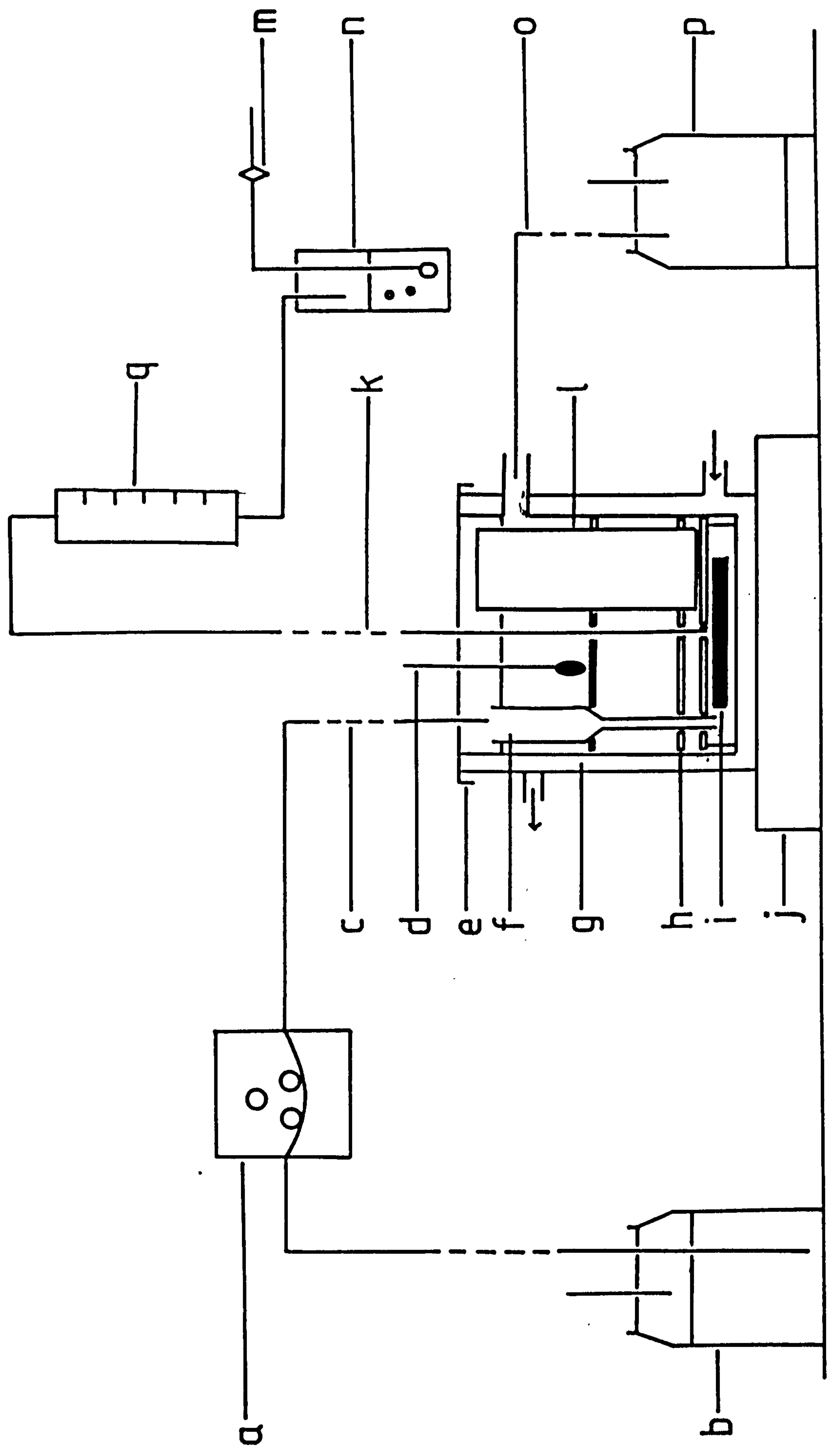
Daily samples were taken to measure the extent of iron oxidation (2.9.1) and the medium flow rates measured and adjusted as required to maintain $70\% \pm 10\%$ of the ferrous iron oxidized.

The level of ferrous iron in the inlet medium was also measured daily.

Sterile chloride was added, as indicated, to the medium pot as a solution of NaCl.

Fig. 2 Diagram of continuous bacterial iron oxidation system.

Key:	a	medium pump	i	stirrer bar
	b	medium reservoir	j	magnetic stirrer unit
	c	medium inlet	k	air inlet tube
	d	thermometer	l	microscope slide
	e	lid	m	air filter
	f	glass funnel	n	air humidifier
	g	water-jacketed vessel	o	medium outlet
	h	perspex carousel slide holder	p	waste pot
			q	air flow meter



2.14 MATERIALS

All chemicals used were of Analar Grade except $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ used for bacterial film iron oxidation experiments (Analar was used in all other work), K_2HPO_4 , NaCl and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ which were Laboratory Grade Reagents. Ceric sulphate was volumetric grade.

Chemicals and reagents were obtained from Fisons Ltd, Loughborough, Leicestershire, with the exception of the following materials.

Acrylamide (electrophoresis grade), N'-N methylene bisacrylamide, phenanthroline-ferrous sulphate complex and sulphur from B.D.H. Ltd., Atherstone, Warwickshire.

Osmium tetroxide, formvar, uranyl acetate and electron microscopy grids from Agar Aids Ltd., Stanstead, Essex.

Bovine albumin, bromophenol blue, 2-mercaptoethanol, N'-N-tetramethyl-ethylenediamine, Trizma-base and Trizma-HCl from Sigma, Poole, Dorset.

Potassium tetrathionate from Fluka (via Fluorochem Ltd., Glossop, Derbyshire).

Agar No.1 from Oxoid, Basingstoke, Hants.

Japanese Agar from Davis Gelatine, Leamington, Warwickshire

Yeast Extract from LAB M, Salford, Manchester.

Carbon Dioxide and air/ CO_2 cylinders from BOC Special Gases, London.

Pyrite and chalcopyrite were kindly provided by N.W. LeRoux, Warren Spring Laboratory, Stevenage, Herts.

P A R T S 3 - 7

RESULTS

P A R T 3

MODERATELY THERMOPHILIC IRON-OXIDIZING BACTERIA

3.1 INTRODUCTION

Acidophilic, moderately thermophilic, rod-shaped, iron-oxidizing bacteria have been isolated from hot springs in Iceland (LeRoux et al., 1977; referred to as Thiobacillus TH1 (Brierley et al., 1978) and thermophile TH1 (Norris et al., 1980)) and Yellowstone National Park (Brock et al., 1976). They have also been isolated from sulphide ore deposits in the U.S.S.R. (Golovacheva & Karavaiko, 1979; designated Sulfobacillus thermosulfidoxidans) and Bulgaria (Groudev et al., 1978), a copper leach dump (Brierley, 1978; designated TH3) and in a test copper-leaching system (Brierley & Lockwood, 1977; designated TH2). All of the isolates were described as optimally active at a temperature of about 50°C and a pH of approximately 2.0 with either ferrous iron or a mineral sulphide as an energy source.

Physiologically, they appeared similar to Thiobacillus ferrooxidans in their ability to oxidize iron and pyrite but required the higher temperature for optimum growth. TH1 oxidized iron at 30°C but at a slower rate than T. ferrooxidans (Brierley et al., 1978; Norris & Kelly, 1978) as its optimum temperature for growth is near 50°C. Although the internal pH of the moderate thermophiles has not been measured that of T. ferrooxidans remains near neutral inside the cell over an external pH range of 1.0 to 8.0 (Cox et al., 1979; Krulwich & Guffanti, 1983). A source of reduced sulphur and organic carbon were demonstrated to be growth requirements for the thermophiles

(Norris et al., 1980); both of which could be supplied by yeast extract. Maximum growth rates occurred when the yeast extract concentration was between 50-200 mg yeast extract per litre with the rate being reduced at higher and lower concentrations (Brierley et al., 1978). Growth on iron was inhibited by glucose (1.0 g.l^{-1}) when in the presence of yeast extract (200 mg.l^{-1}) (Brierley et al., 1978). Growth also occurred if glutathione ($2-20 \text{ mg.l}^{-1}$) replaced the yeast extract but the rate of iron oxidation was slower than with optimum yeast extract concentrations (Norris et al., 1980). A slow rate of Fe^{2+} oxidation has been demonstrated when sodium thiosulphate (100 mg.l^{-1}) or potassium tetrathionate (100 mg.l^{-1}) replaced the yeast extract and no organic source of carbon was added (Brierley et al., 1978). Several studies, however, have shown that TH1 was unable to fix more than 4% of its cell carbon from CO_2 (Brierley et al., 1978; Brierley et al., 1980). It has therefore been described as a chemolithotrophic heterotroph (Whittenbury & Kelly, 1977; Kelly, 1981) obtaining energy from the inorganic oxidation of ferrous iron and carbon from an organic source. In contrast, T. ferrooxidans is a chemolithotrophic autotroph as it can obtain all of its carbon from CO_2 .

Morphologically, when grown on a ferrous iron-yeast extract medium, the moderate thermophiles have appeared to vary in size from $1.6-3.2 \text{ }\mu\text{m}$ by $0.8 \text{ }\mu\text{m}$ for TH1 (Brierley, 1978), $1.0-3.0 \text{ }\mu\text{m}$ by $0.6-0.8 \text{ }\mu\text{m}$ for S. thermosulfidooxidans (Golovacheva & Karavaiko,

1978) to the narrower TH3 measuring 1.1-1.6 μm by 0.5 μm (Brierley, 1978). The presence of a flagellum has been observed in an isolate from a copper ore body (Berry et al., 1978b; Berry & Murr, 1980). S. thermosulfidooxidans has been reported to produce spores resistant to sterilization at 110°C for 30 minutes (Golovacheva & Karavaiko, 1978).

The DNA base composition from ferrous iron-yeast extract grown cells was 48 mol % guanine + cytosine for TH1 (Brierley et al., 1978) and 53.6-56.9 mol % for S. thermosulfidooxidans (Golovacheva & Karavaiko, 1978). The DNA from T. ferrooxidans contains 58.3 mol % GC (Brierley et al., 1978).

Manometric experiments with non-growing cell suspensions of TH1 have shown that oxygen uptake and hence respiration rate on ferrous iron increased over the pH range 1.3 to 3.2. Oxygen uptake also increased as the ferrous iron concentration increased up to 81 mM Fe^{2+} but then remained the same at 111 mM Fe^{2+} (Brierley & LeRoux, 1977).

Thermophiles TH1 and TH3 have been grown heterotrophically on yeast extract (Norris et al., 1980) but not on glucose or fructose (Brierley et al., 1978). S. thermosulfidooxidans was capable of heterotrophic growth on glucose or sucrose after a period of adaptation (Golovacheva & Karavaiko, 1978).

The aim of this work with moderately thermophilic iron-oxidizing bacteria was to isolate organisms from a range of sites, with the emphasis on obtaining autotrophic, thermophilic,

iron-oxidizing organisms and then to compare the organisms with a view to a preliminary classification of the 'group' and an assessment of their potential use in applied biohydrometallurgy.

3.2 SELECTION OF A GROWTH MEDIUM

Growth of thermophile TH1 on 50 mM Fe^{2+} supplemented with yeast extract (0.2 gl^{-1}) at 50°C in four different media showed no significant differences in the rate of iron oxidation with the different salt concentrations (Fig.3). However the media of Leathen and Norris produced a shortening in the length of the exponential phase of growth with the result that incomplete iron oxidation occurred. In the higher salt concentrations of Tuovinen and Kelly and Silverman and Lundgren there was extensive precipitation of iron and salts. Therefore a salts medium containing (gl^{-1}), $(\text{NH}_4)_2\text{SO}_4$ (0.2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4), KCl (0.1) and K_2HPO_4 (0.1) was selected as it allowed complete iron oxidation to occur without excessive precipitation. This was referred to as the ferrous iron medium.

3.3 ISOLATION OF MODERATELY THERMOPHILIC IRON-OXIDIZING BACTERIA

Organisms from ferrous iron enrichment cultures, grown in the absence of organic nutrients, from Kingsbury, Evenwood, Birch Coppice and Lake Myvam were purified on ferrous iron plates (see Section 2.7.1) at 50°C by single colony isolation.

Organisms from ferrous iron enrichment cultures grown in the presence of yeast extract from the above sites were purified

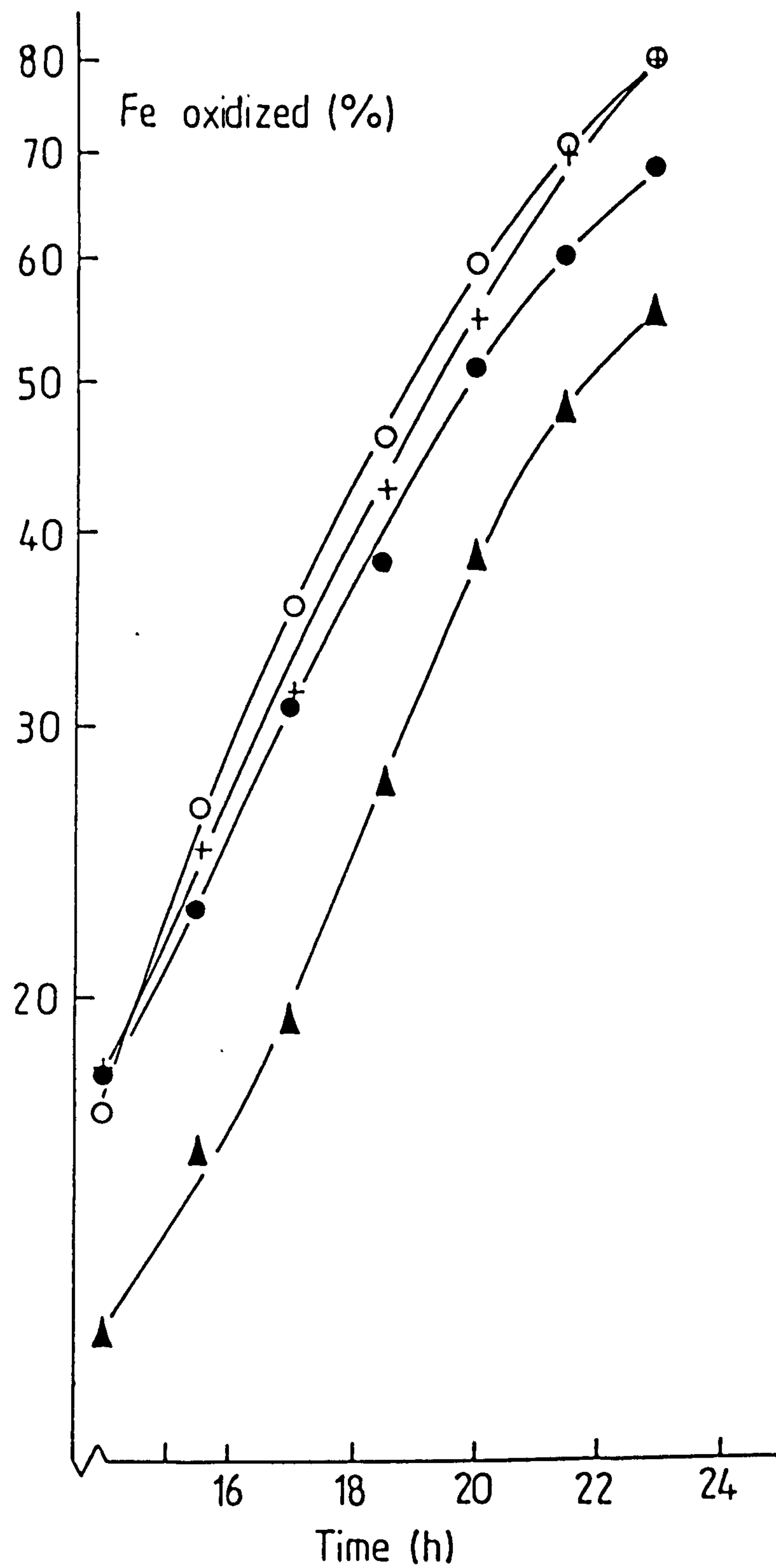


Fig. 3 Ferrous iron oxidation during growth of thermophile TH1 at 50°C in the presence of yeast extract (0.2 gl^{-1}) in the salts media of Leathen (▲), Norris (●), Tuovinen and Kelly (○) and Silverman and Lundgren (+).

at 50°C on yeast extract supplemented ferrous iron plates and yeast extract plates (see Section 2.7.3) by single colony isolation.

An organism from an autotrophic iron enrichment culture at 37°C from Alvecote was isolated and purified by P. Norris.

Enrichment cultures growing on ferrous iron and on pyrite at 50°C in the presence of yeast extract from Alvecote and a Canadian (INCO Ltd) nickel mine were not purified and are referred to as enrichment cultures of the sample sites. Similarly, an enrichment culture from Kingsbury, growing on ferrous iron at 60°C in the presence of yeast extract, was not purified and is referred to as the 60°C Kingsbury enrichment culture to distinguish it from other pyrite enrichments of samples from the Kingsbury site.

3.4 GROWTH ON FERROUS IRON

Chemolithoheterotrophic growth of thermophile TH1 measured as the rate of ferrous iron oxidation followed the sigmoidal curve characteristic of bacterial growth (Fig.4). This was accompanied by a rise in pH from 1.7 to 2.05 which caused some of the ferric iron produced to precipitate.

Measurement of the growth rate on ferrous iron by determinations of iron oxidation, protein, cellular carbon and optical density showed that all methods were reliable indicators of growth during the early stages of a growth curve (Fig.5). Protein analysis was only reliable during the early phase of

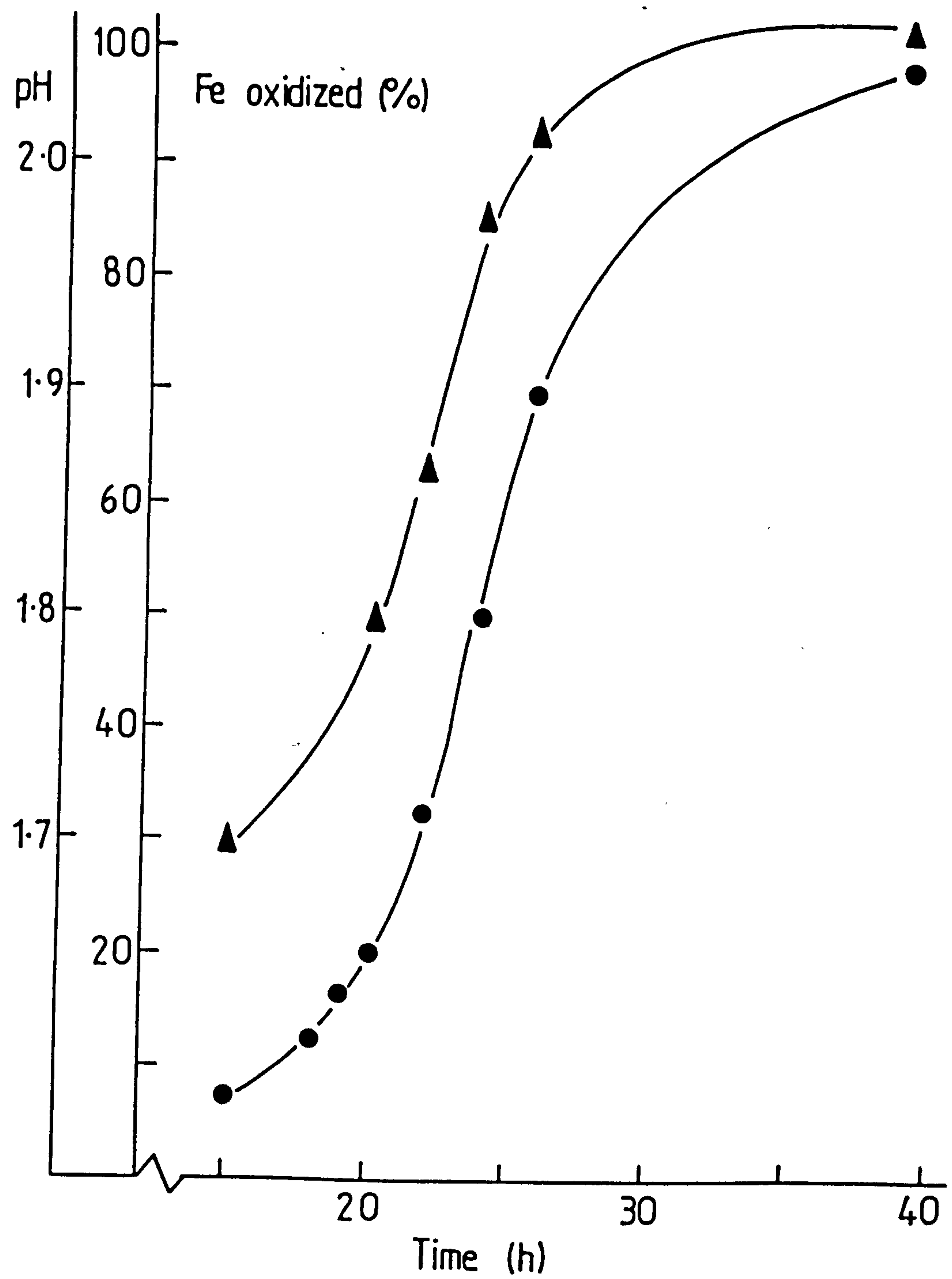


Fig. 4 The pH (▲) of the yeast extract (0.2g l^{-1}) -supplemented ferrous iron medium during growth and iron oxidation (●) by thermophile TH1.

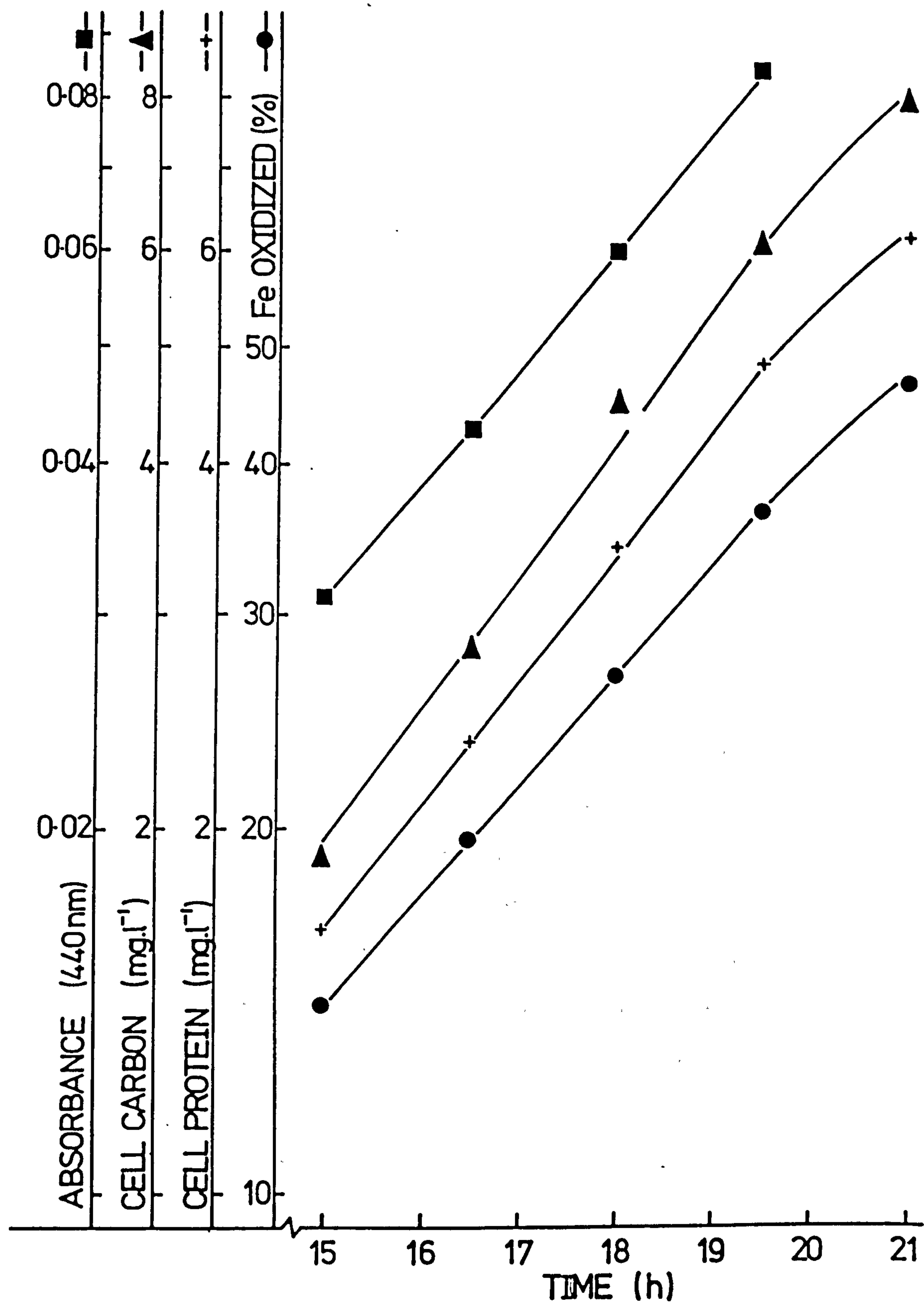


Fig. 5 Iron oxidation during growth of thermophile TH1 at 50°C in the ferrous iron medium supplemented with yeast extract.

exponential growth as the ferric iron precipitated due to the pH of the medium increasing during growth caused an interference in the analysis. Increasing the number of washings of the pellet did not produce a cleaner pellet for analysis (Fig.6) but some of the protein was lost with the washing. The measurement of optical density was also only reproducible during the early phase of exponential growth as the ferric iron interfered with readings when it precipitated.

TH1, expressed as a percentage of dry weight, was composed of 42.42% carbon, 6.25% hydrogen and 9.3% nitrogen. With the same preparation and analysis procedures, T. ferrooxidans contained 42.15% carbon, 6.49% hydrogen and 9.42% nitrogen.

The influence of temperature on growth rate on ferrous iron was determined by measuring the rate of iron oxidation over the period when 10-20% of the available iron was being oxidized. Exponential growth was not always maintained as the ferrous iron was progressively oxidized, possibly because of ferric iron end product inhibition or because of the falling concentration, by utilization or decomposition, of the reduced sulphur supplement. In the presence of yeast extract, the optimum temperature for all isolates was 50°C although the rate varied from a doubling time for iron oxidation of 1.6 hours for the Birch Coppice isolate to 5.0 hours for the Kingsbury isolate (Fig.7). Grown chemolitho-autotrophically on ferrous iron TH1, the Evenwood and Lake Myvam isolates had lower optimum temperatures for growth of 45°C while

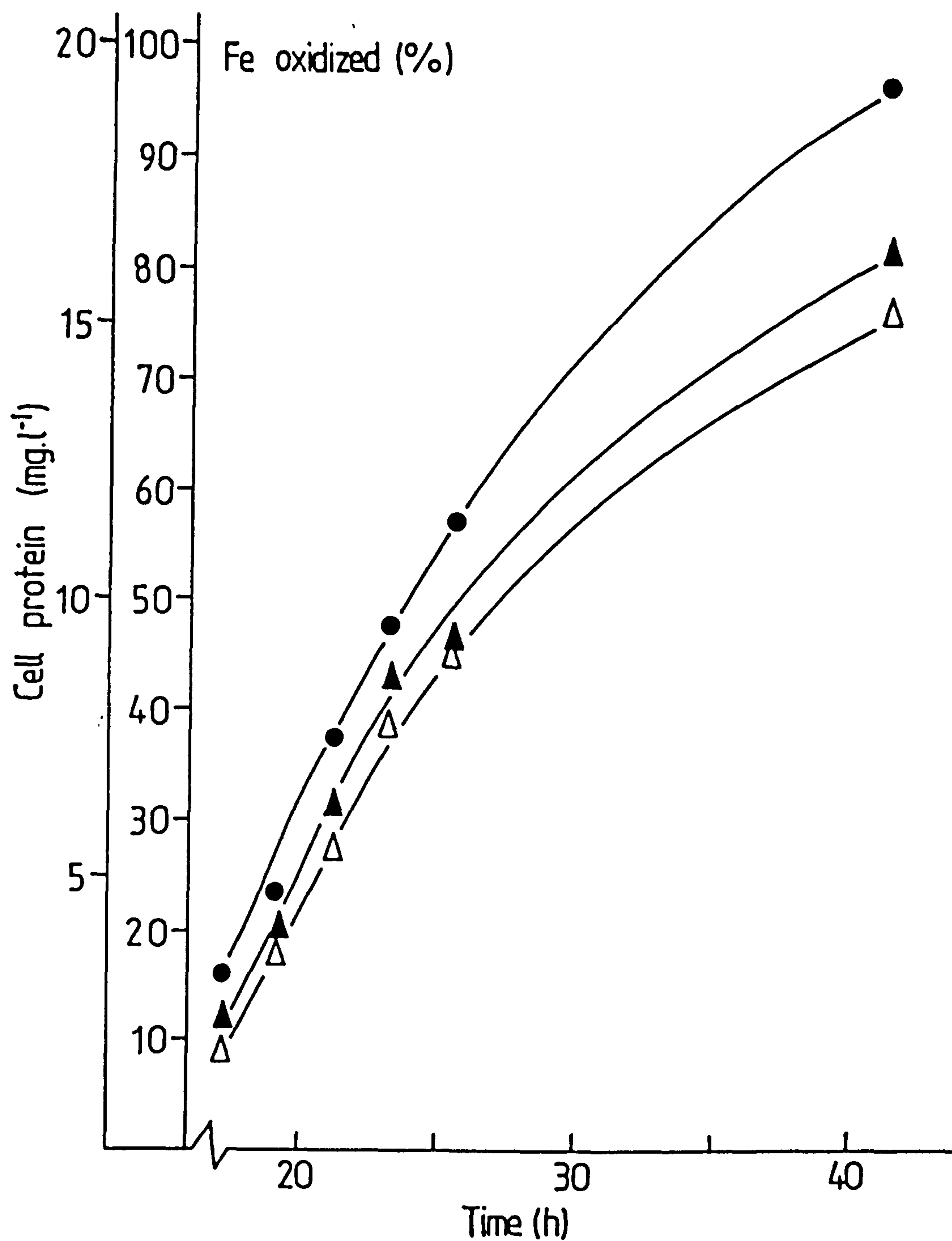


Fig. 6 Iron oxidation (●) by TH1 at 50°C in the ferrous iron medium supplemented with yeast extract (0.2gl^{-1}). Culture cell protein concentrations were determined as described in the Materials and Methods with the modification of one (▲) and four (Δ) washes of cell pellets with 4 ml volumes of acidified distilled water.

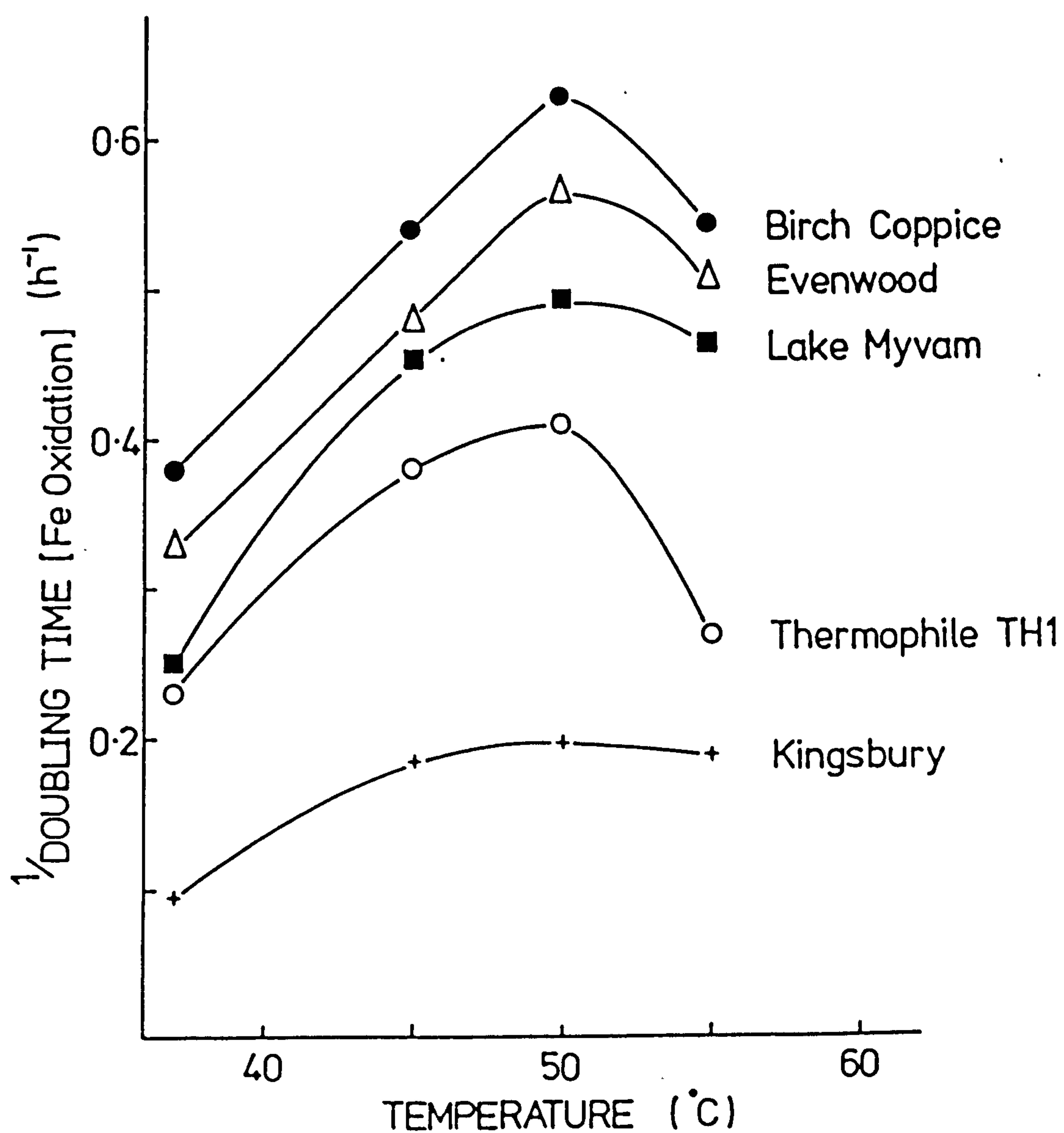


Fig. 7 The effect of temperature on growth-associated iron oxidation by thermophile TH1 and other moderate thermophiles (sites of isolations indicated above) in yeast extract (0.2g.l^{-1}) - supplemented ferrous iron medium. 0.5% (v/v) inocula were used.

that of the Birch Coppice and Kingsbury isolates remained at 50°C (Fig.8). TH1 had been maintained in the presence of yeast extract since 1977 before this demonstration of autotrophic growth. The maximum rate of iron oxidation varied from a doubling time of 7.0 hours for the Birch Coppice isolate to 16.0 hours for TH1. With the exception of the Kingsbury isolate, the rate of iron oxidation was approximately 5 times faster in the presence of yeast extract. The Kingsbury isolate only showed a 1.5 times improvement in the presence of yeast extract. Under these conditions there was no measurable growth by any of the moderate thermophiles at 60°C. However the Birch Coppice isolate was grown at 60°C after a further 18 months maintenance in the laboratory on the ferrous iron medium.

An enrichment culture of rod-shaped, iron-oxidizing bacteria was isolated at 60°C on ferrous iron from Kingsbury. When the pH of the ferrous iron medium was lowered to 1.6 and an inoculum size of 5% (v/v) was used, measurable growth by TH1 could also be obtained at 60°C to compare with the Kingsbury enrichment culture (Fig.9). The Kingsbury enrichment culture had a doubling time of 2.5 hours compared with the slower time of 5.5 hours obtained with TH1.

The effect of temperature and pH on ferrous iron oxidation by the Evenwood isolate and the 60°C Kingsbury enrichment culture showed that the rate of iron oxidation was faster at the pH of 1.6 than 2.0 at temperatures from 50° - 60°C (Fig.10). The Evenwood isolate was unable to grow at 60°C and the Kingsbury

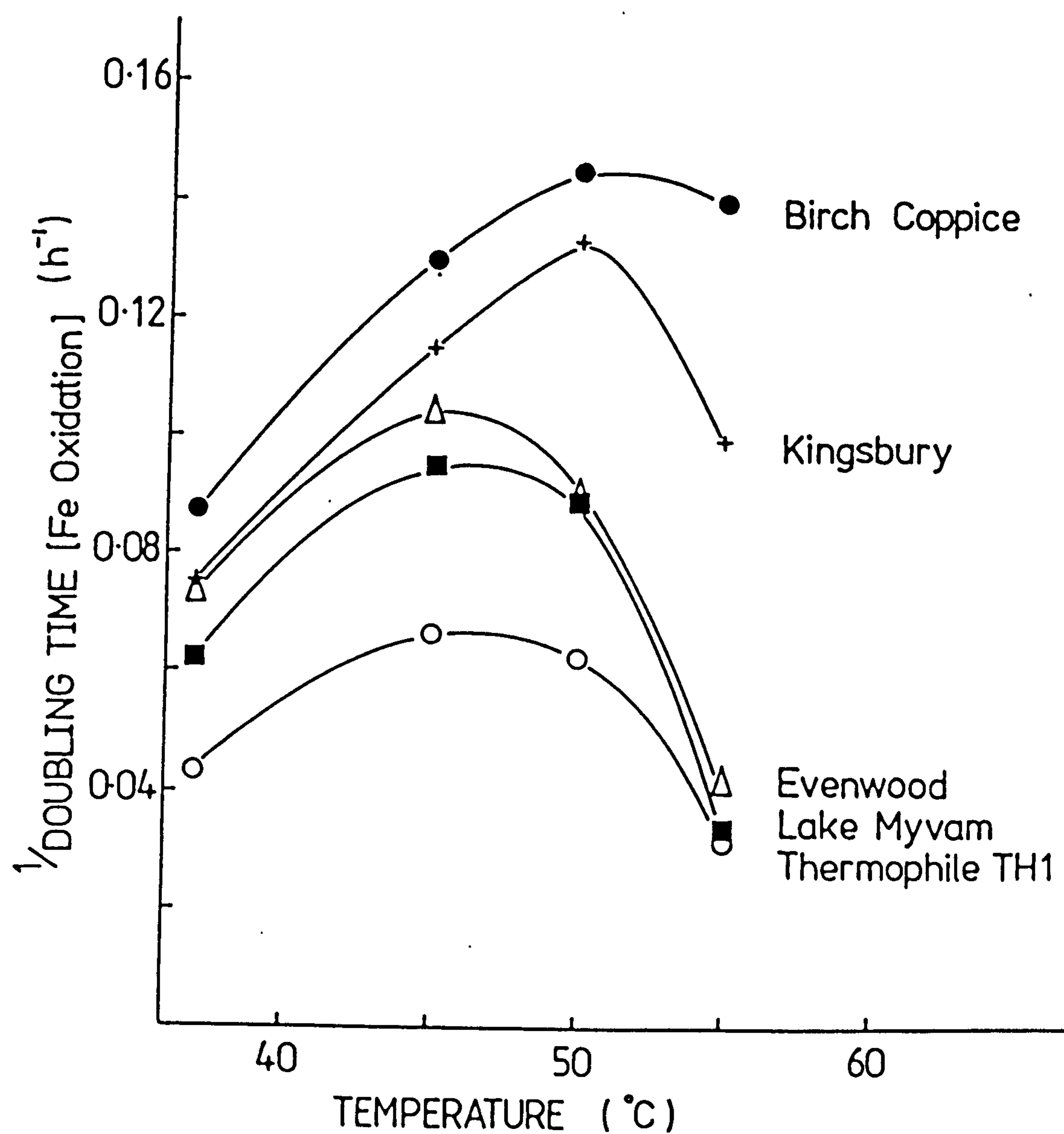


Fig. 8 The effect of temperature on growth-associated iron oxidation by thermophile TH1 and other moderate thermophiles (sites of isolations indicated above) in sodium thiosulphate (0.2g.l^{-1}) -supplemented ferrous iron medium and with a CO_2 -enriched (5% v/v in air) atmosphere. 2% (v/v) inocula were used.

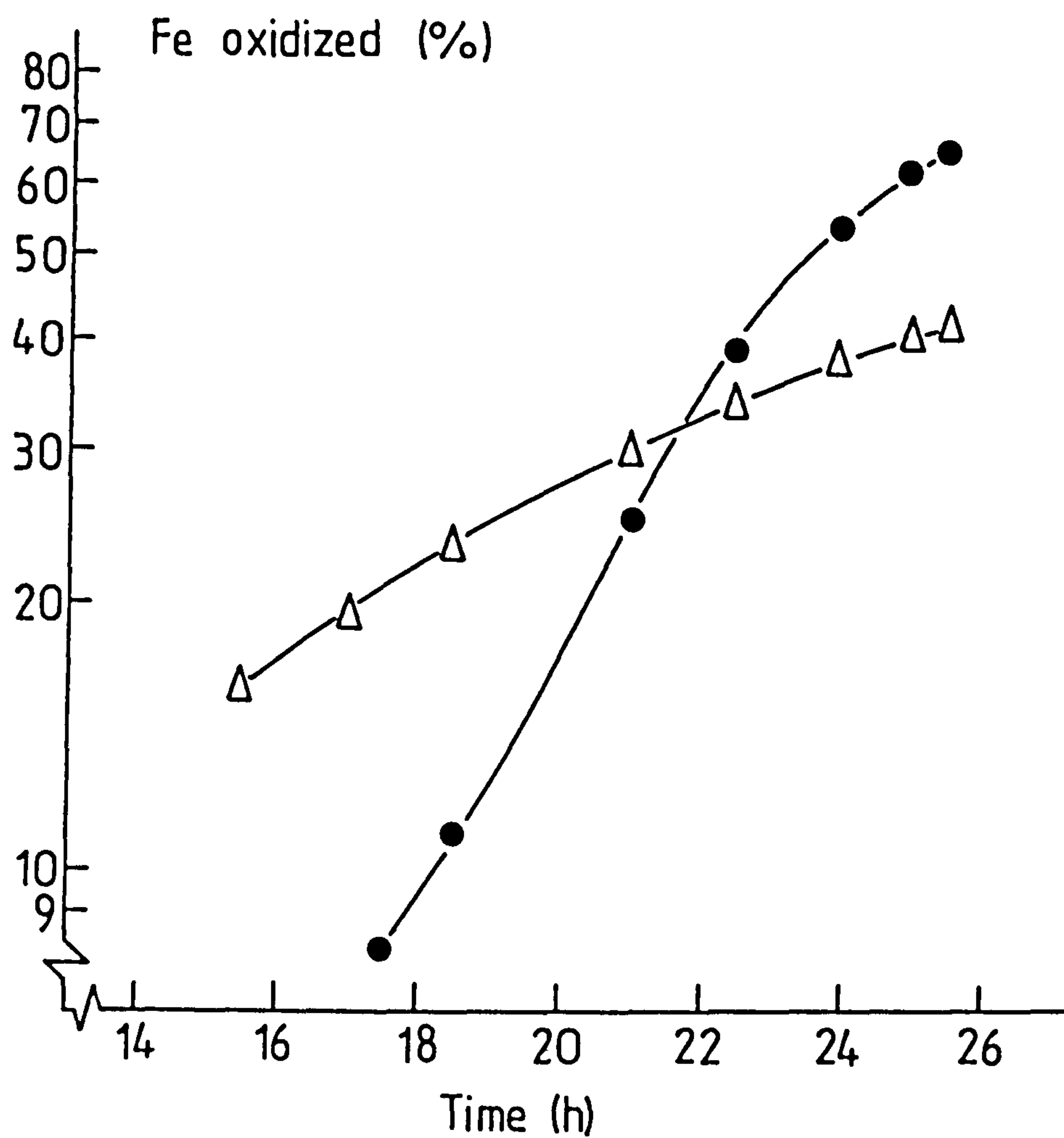


Fig. 9 Ferrous iron oxidation during growth of thermophile TH1 (Δ) and the Kingsbury 60°C enrichment culture (●) in the yeast extract (0.2gl^{-1})-supplemented ferrous iron medium at 60°C.

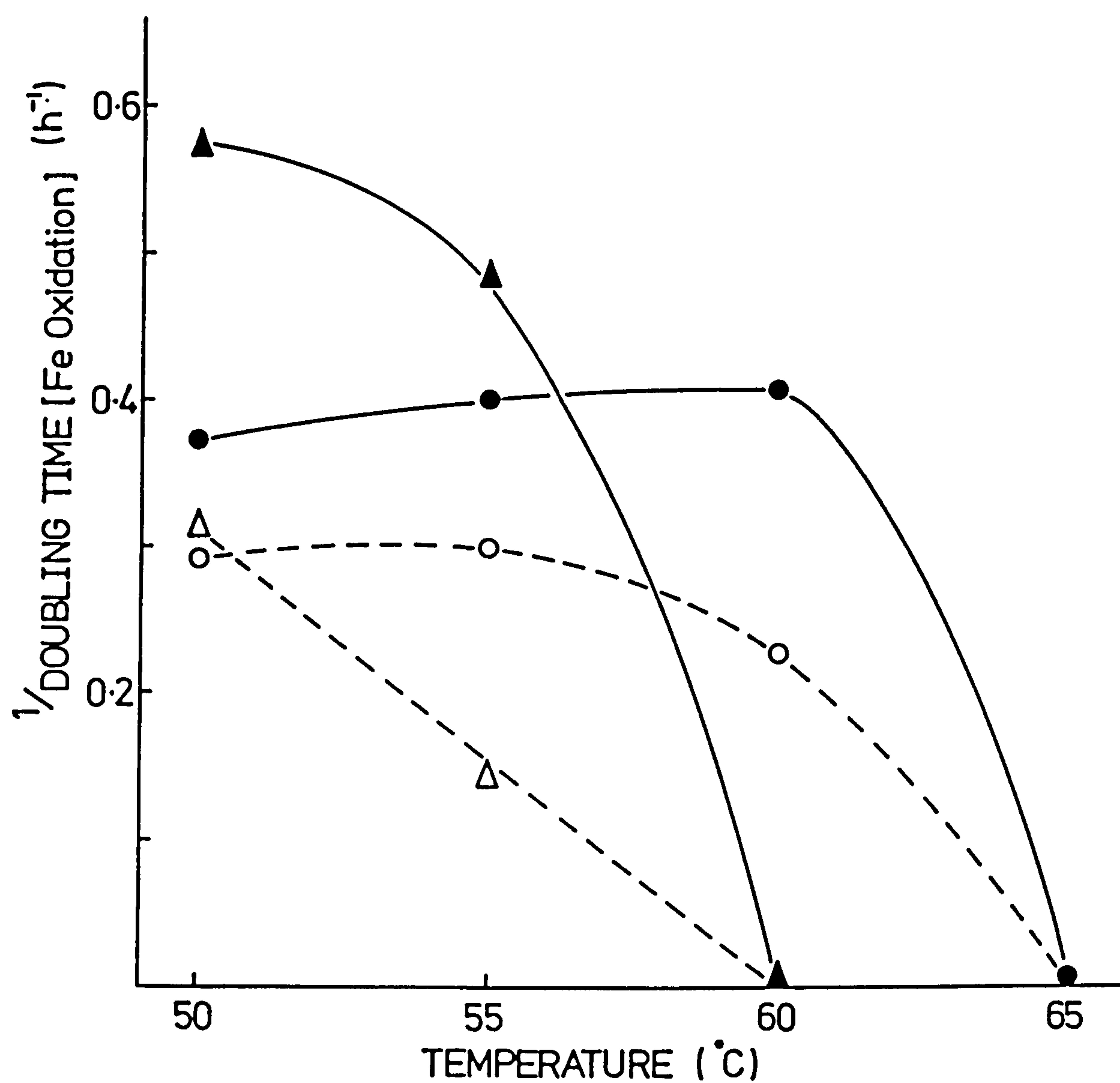


Fig. 10 The effect of temperature and pH on iron oxidation during growth of the Evenwood isolate at pH 1.6 (▲) and 2.0 (△) and of the Kingsbury 60°C enrichment culture at pH 1.6 (●) and 2.0 (○) in the ferrous iron medium supplemented with yeast extract (0.2gl^{-1}).

enrichment culture unable to grow at 65°C at either of the pH values.

The autotrophic rate of iron oxidation by the Birch Coppice isolate was similar whether a 5% (v/v) or 20% (v/v) CO₂ in air mixture was used. However when the flask was continuously flushed with air there was a two-fold reduction in rate (Fig.11) indicating that a level of carbon dioxide above atmospheric was necessary for optimum growth.

The thiosulphate added to the ferrous iron medium was unstable at the experimental pH and was added solely to provide a source of reduced sulphur. With no addition of thiosulphate to the medium, although a trace would have been present due to carry over in the inoculum, growth of the Birch Coppice isolate was slow during the entire growth phase (Fig.12). The addition of thiosulphate, even at low concentrations, increased the rate of iron oxidation. At higher thiosulphate concentrations (0.02-0.2 g l⁻¹) the rate of iron oxidation did not appear to increase but the duration of the higher rate of oxidation during the exponential phase was extended.

Enrichment cultures on iron in the presence of yeast extract at 50°C frequently contained very short, non-motile, rod-shaped bacteria as well as the larger, moderately thermophilic iron-oxidizers. An example of the short rod-shaped bacteria was purified from the Kingsbury enrichment culture on yeast extract agar plates. It was unable to oxidize iron but

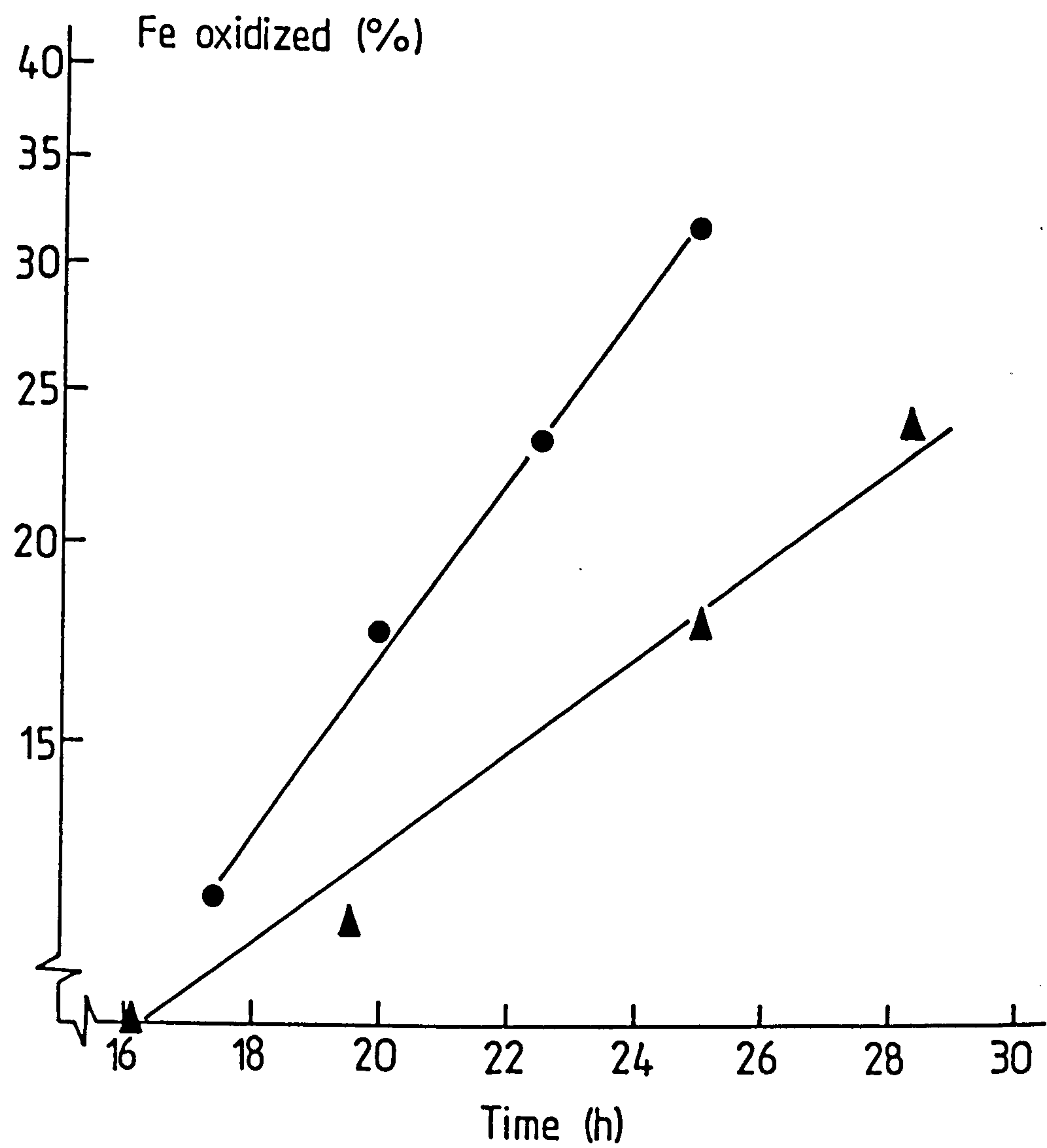


Fig. 11 Ferrous iron oxidation during autotrophic growth of the Birch Coppice isolate at 50°C with flask gas atmospheres of air (▲) and 5% (v/v) CO₂ in air (●).

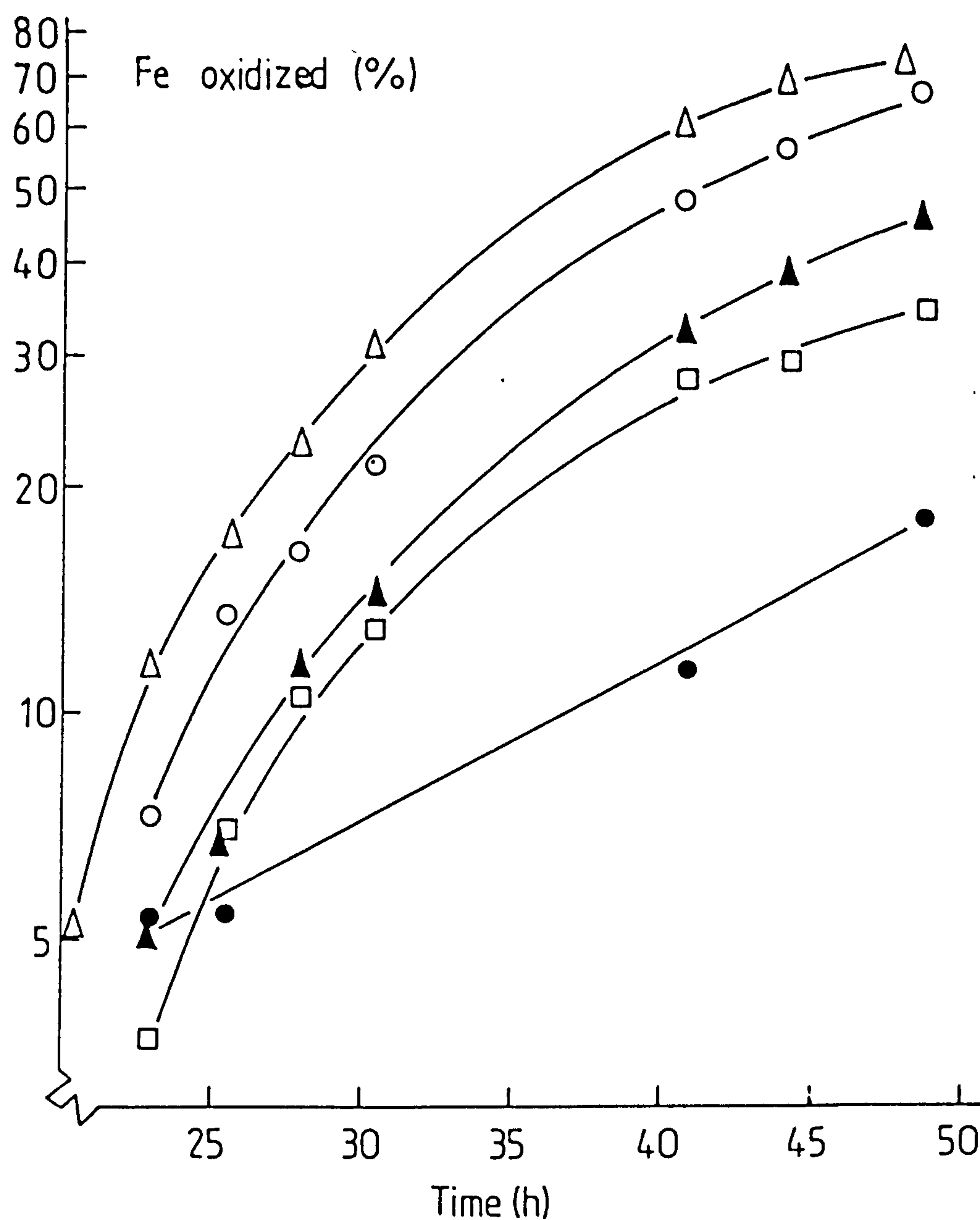


Fig. 12 The effect of thiosulphate concentration on ferrous iron oxidation by the Birch Coppice isolate. The flask atmospheres were enriched to 5% (v/v) CO_2 in air and the ferrous iron medium was supplemented with $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ to concentrations of (g.l^{-1}) 0.01 (□), 0.02 (▲), 0.1 (○), 0.2 (△) or received no thiosulphate (●).

grew rapidly on yeast extract. When the 50°C Kingsbury enrichment culture was grown at pH 2.0 in yeast extract-supplemented ferrous iron medium, the heterotroph grew on the yeast extract and inhibited iron oxidation by the moderate thermophile. At lower pH values, growth of the heterotroph was suppressed and iron oxidation occurred more rapidly. These observations were made by visual assessment of ferrous to ferric iron conversion in shake flask cultures and growth rates were not quantitatively determined.

3.5 HETEROTROPHIC GROWTH ON YEAST EXTRACT

TH1 grew heterotrophically in a yeast extract medium (see Section 2.4.2) at 50°C with concentrations of 0.1 to 0.5g yeast extract per litre (Fig.13). Maximum optical density occurred with 0.5g yeast extract l^{-1} although there was a longer lag phase than that observed at lower concentrations. A level of 1.0g yeast extract per litre was inhibitory to growth. A decrease in optical density occurred once the maximum had been reached indicating that the cells had metabolised the available yeast extract and had begun to lyse. The pH remained at 1.8 during the growth on yeast extract.

When measured by optical density and cellular protein the doubling time for TH1 growing on yeast extract ($0.2g l^{-1}$) was 8.0 hours (Fig.14). This was approximately 3 times slower than that observed for growth on iron in the presence of yeast extract (Fig.7).

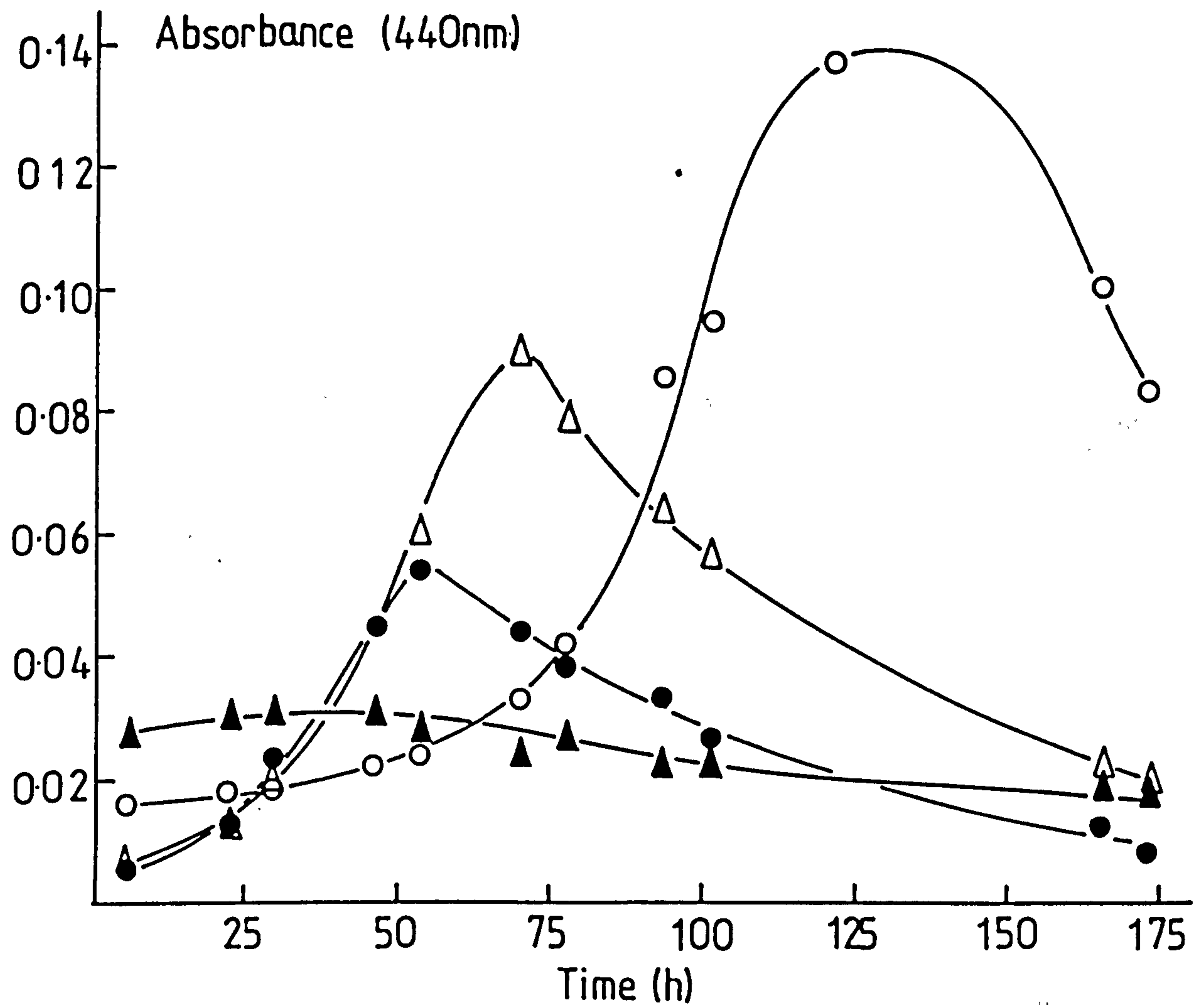


Fig. 13 Heterotrophic growth of TH1 at 50°C with initial yeast extract concentrations (g.l^{-1}) of 0.1 (●), 0.2 (△), 0.5 (○) and 1.0 (▲).

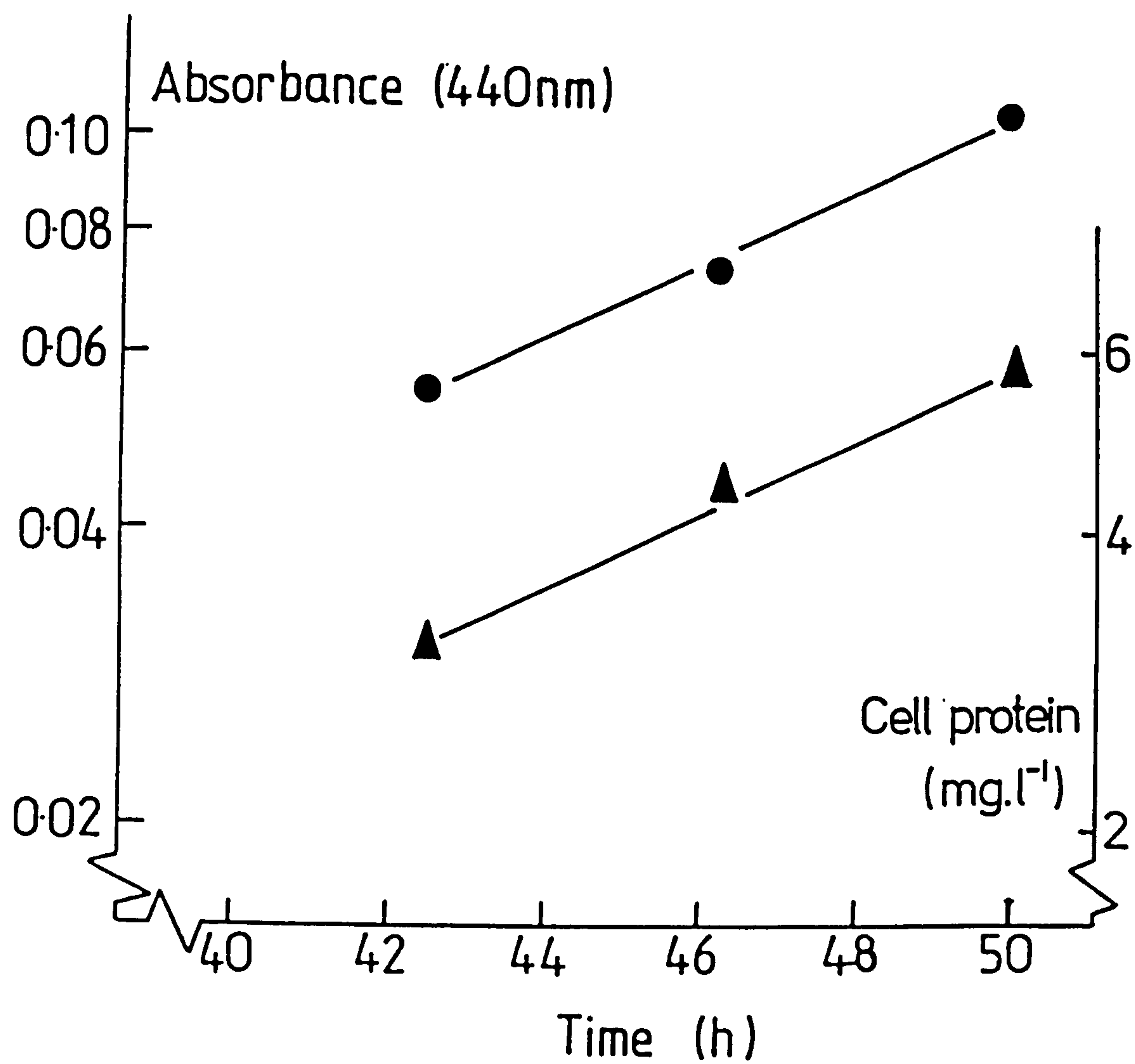


Fig. 14 Semi-logarithmic plot of culture optical density (●) and cell protein (▲) during growth of TH1 on yeast extract (0.2g.l⁻¹) at 50°C.

TH1 was serially sub-cultured 10 times on yeast extract before its growth on ferrous iron was compared with an iron grown culture (Fig.15). There was no loss of iron-oxidizing ability by the yeast extract-grown cells during this time.

3.6 MORPHOLOGY

Under the light microscope, differences were observed in the morphology of the isolates which were dependent on their growth substrate and type of growth. When grown on ferrous iron in the presence of yeast extract, all of the isolates appeared as long, fat, rod-shaped bacteria. When grown autotrophically their size was reduced. Electron micrographs illustrate these morphological variations. TH1 grown on iron in the presence of yeast extract was 1.7-3.8 μm by 1.0 μm but when grown autotrophically its size was reduced to 1.6-2.2 μm by 0.5 μm (Fig.16). Similar variations occurred with the Birch Coppice isolate which measured 2-3.2 μm by 1.0 μm in the presence of yeast extract and 1.8-2.2 μm by 0.7 μm in its absence (Fig.17). Under the same conditions at 30°C T. ferrooxidans measured, whether grown in the presence or absence of yeast extract, 0.9-1.5 μm by 0.5 μm (Fig.18) and was generally smaller than the moderate thermophiles.

When TH1 was grown on yeast extract alone it appeared as short chains of almost spherical organisms surrounded by an optically refractile coat. The cells measured 0.8-1.6 μm by 0.8-1.0 μm (Fig.19). The outer layer, which was clearly visible

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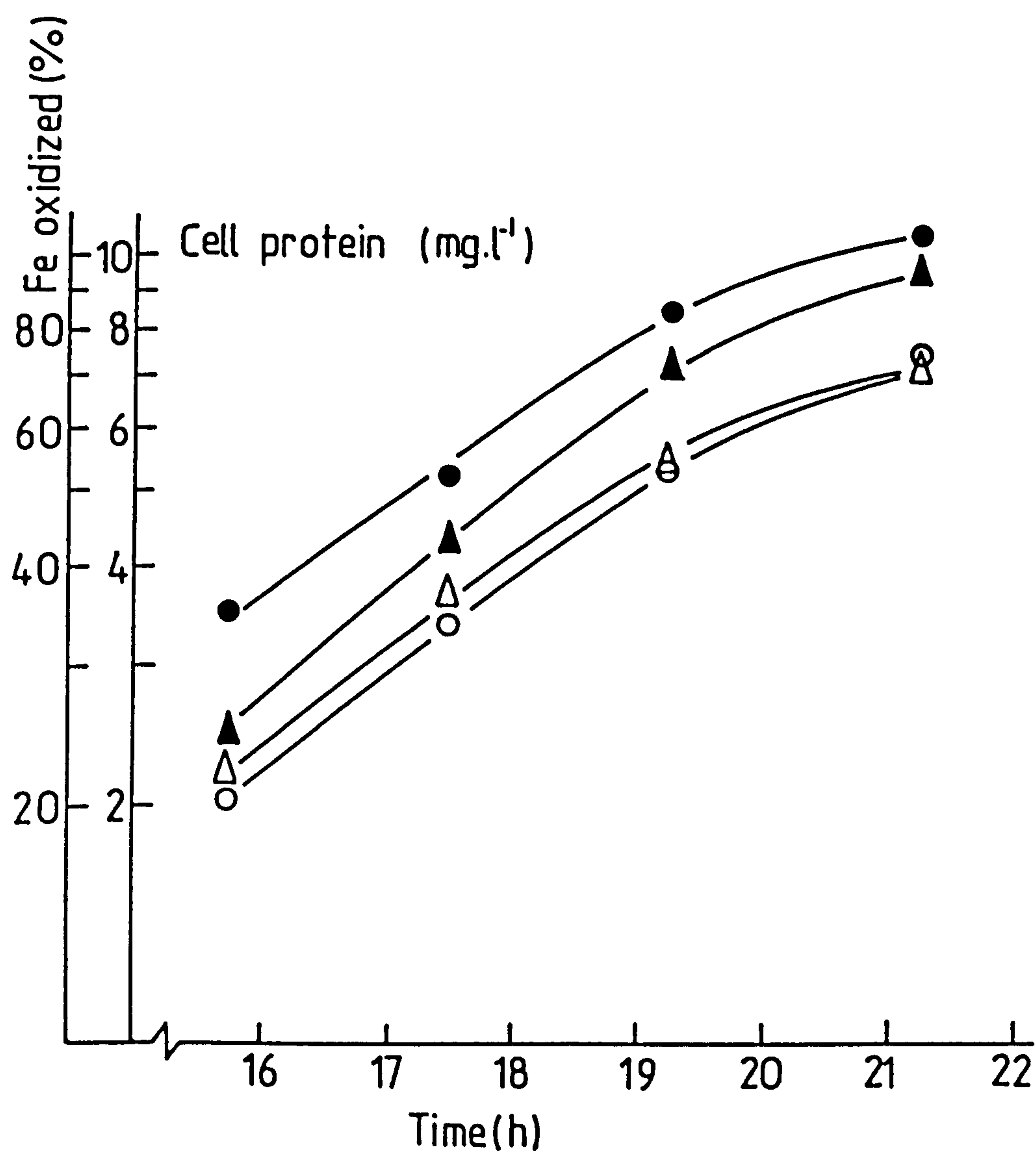


Fig. 15 Ferrous iron oxidation by TH1 in yeast extract (0.2g.l⁻¹) -supplemented ferrous iron medium at 50°C following the use of iron-grown (○) and yeast extract-grown (△) inocula. The corresponding culture cell protein concentrations (●, ▲) are also shown.

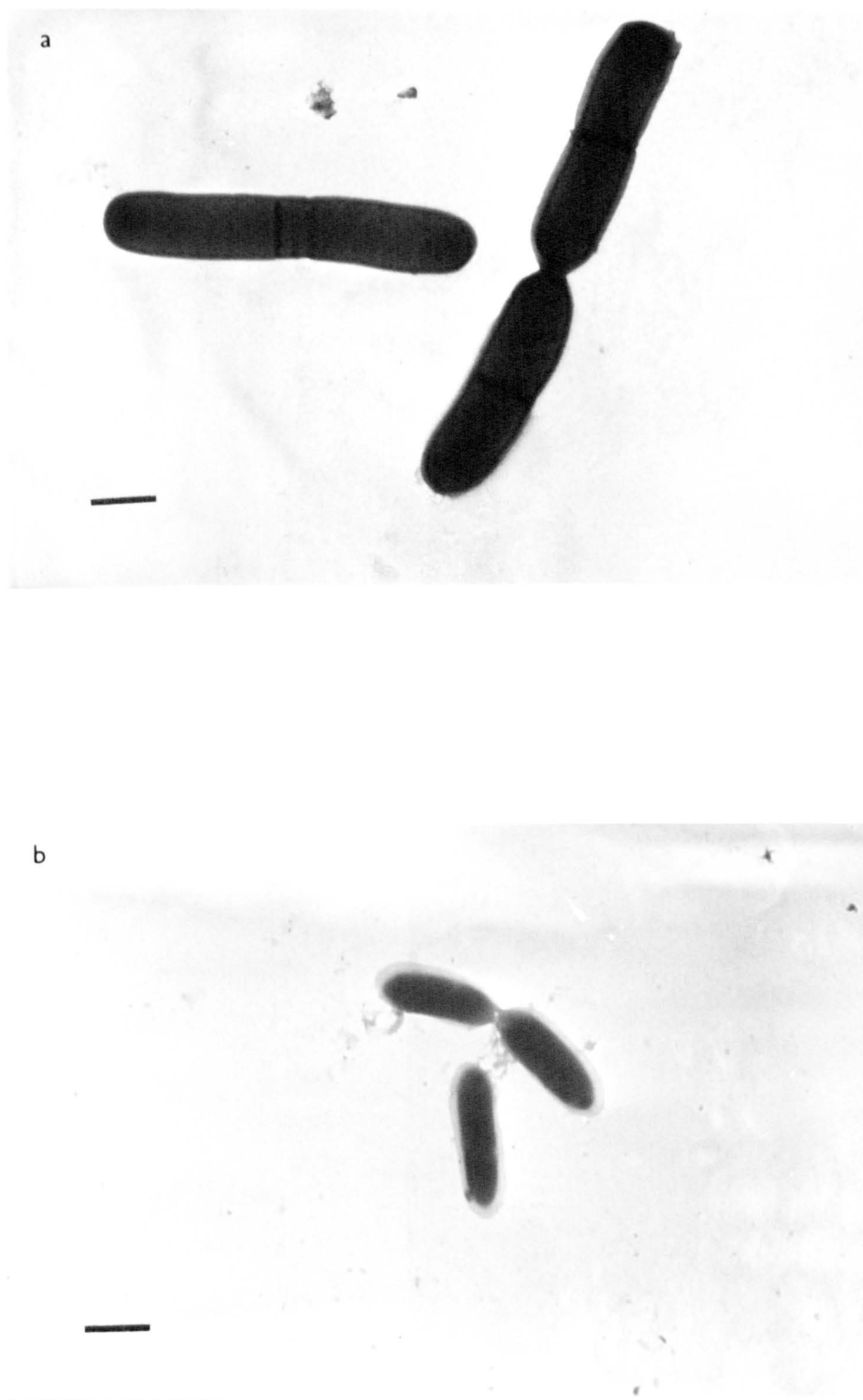


Fig. 16 Thermophile TH1 grown on ferrous iron (a) in the presence of yeast extract (0.2g.l^{-1}) and (b) autotrophically. The bar marker represents $1\text{ }\mu\text{m}$.

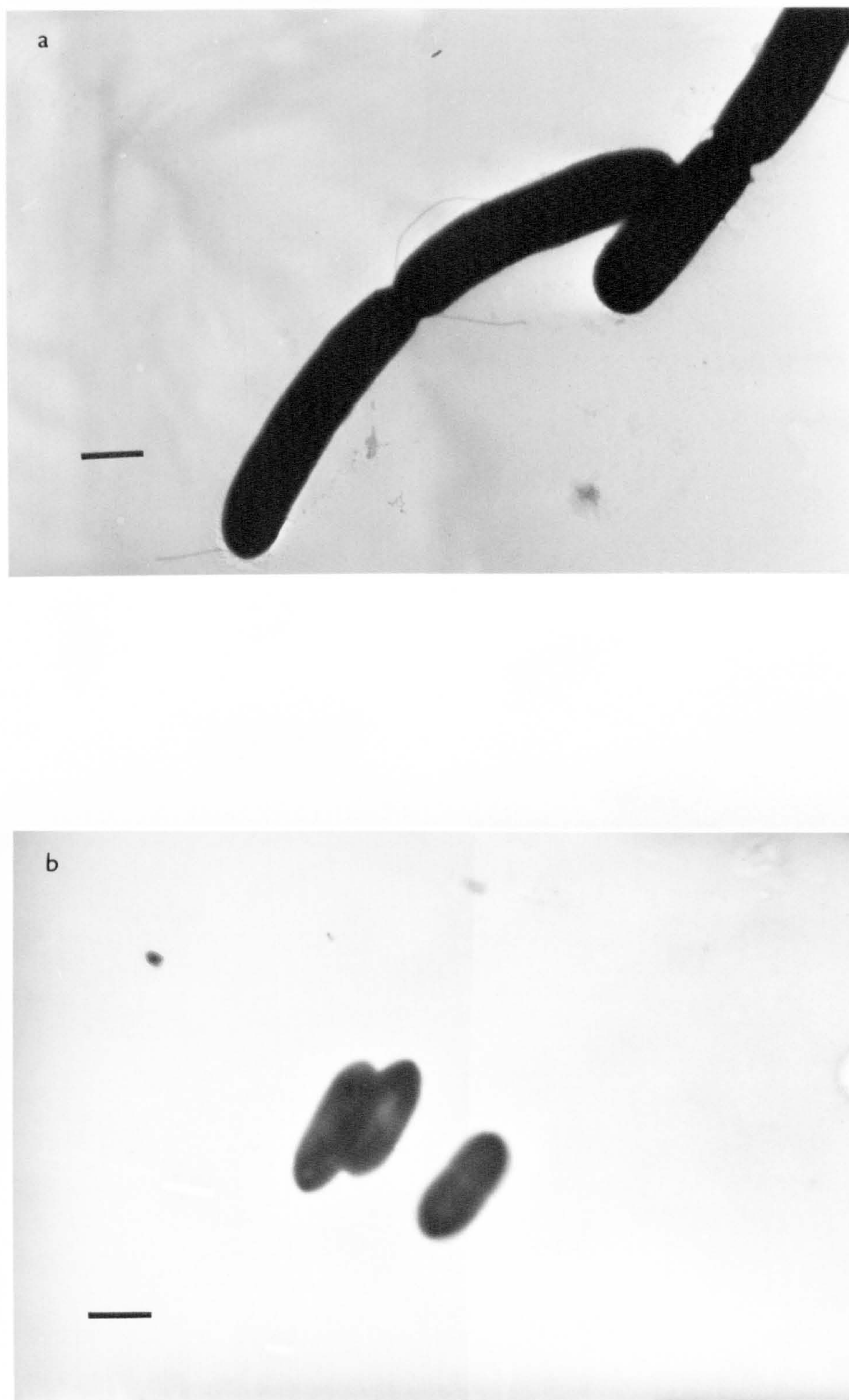


Fig. 17 The Birch Coppice isolate grown on ferrous iron (a) in the presence of yeast extract (0.2g.l^{-1}) and (b) autotrophically. The bar represents $1\text{ }\mu\text{m}$.

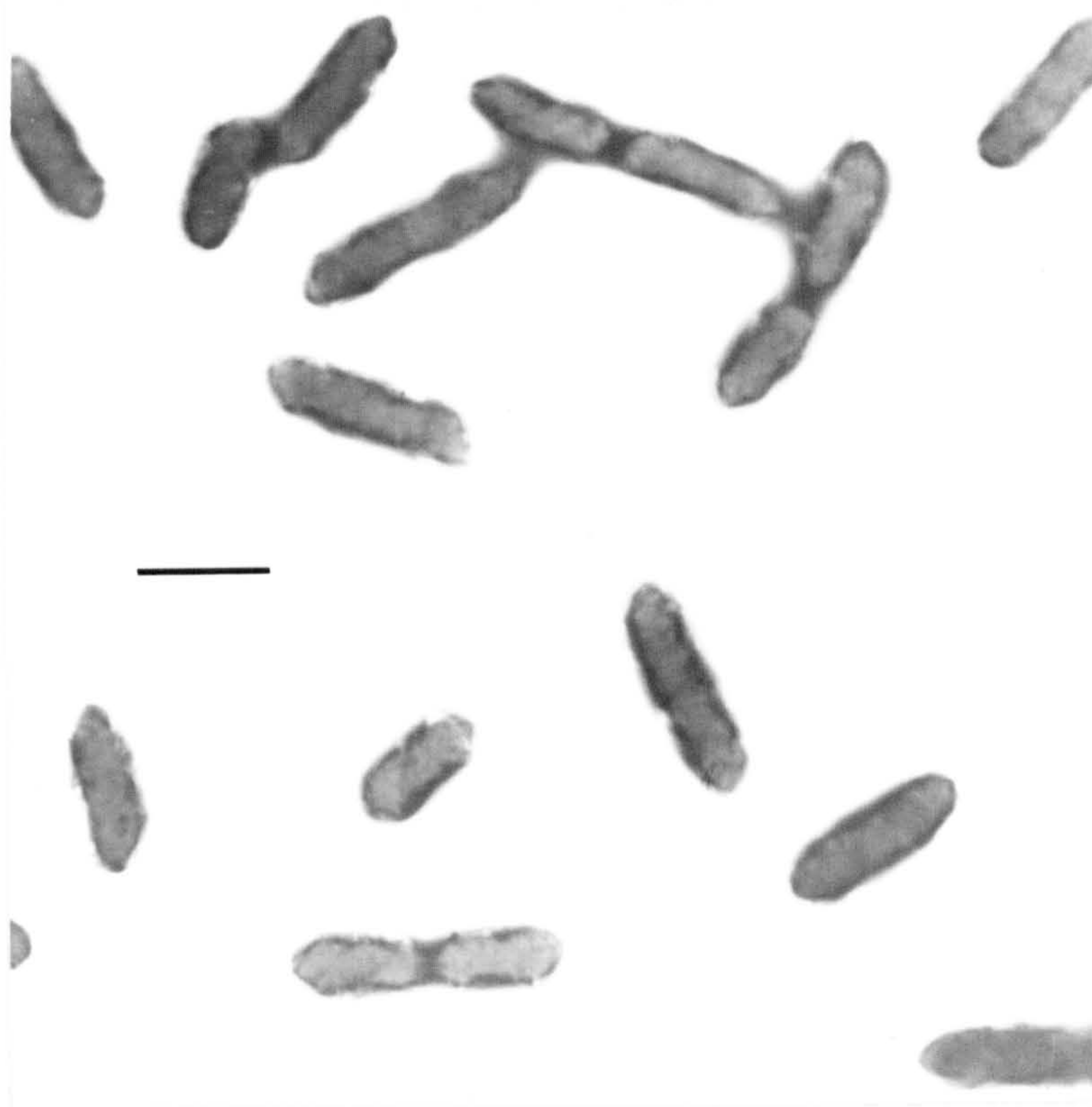


Fig. 18 Thiobacillus ferrooxidans grown autotrophically on ferrous iron. Bar marker, 1 μm .



Fig. 19 Thermophile TH1 grown heterotrophically on yeast extract (0.2g.l^{-1}). Bar marker, $1\text{ }\mu\text{m}$.

under the light microscope, was not easily detected under the electron microscope due, presumably to the dry nature of the preparation.

The obligately heterotrophic bacterium isolated at 50°C from the Kingsbury enrichment culture was smaller than the moderate thermophiles when grown on iron in the presence of yeast extract, being 1.0–2.4 μm x 0.6 μm in size (Fig.20).

3.7 COMPARISON OF ISOLATES BY ELECTROPHORESIS OF CELL PROTEIN LYSATES

To compare the new isolates with TH1, 10 of the major protein bands of TH1, i.e. 10 of the darker bands on the electrophoresis gel, were selected (—) as reference bands (Fig.21). When grown chemolithoheterotrophically on iron (Fig.21), 8 out of the 10 protein bands of TH1 appeared in identical positions in the Evenwood, Lake Myvam, Kingsbury and Birch Coppice isolates. Two of the bands (—†) in the new isolates appeared slightly lower than in TH1. The Alvecote isolate showed a very different cell protein pattern with only one major protein band in common with TH1 and the other isolates (—) and one with the other isolates (—†). As expected, T. ferrooxidans also showed a completely different cell-protein pattern to TH1 and the Alvecote isolate with only two major protein bands (<) in similar positions.

When grown chemolithoautotrophically on ferrous iron (Fig.22) 7 of the major protein bands of TH1 (—) were selected

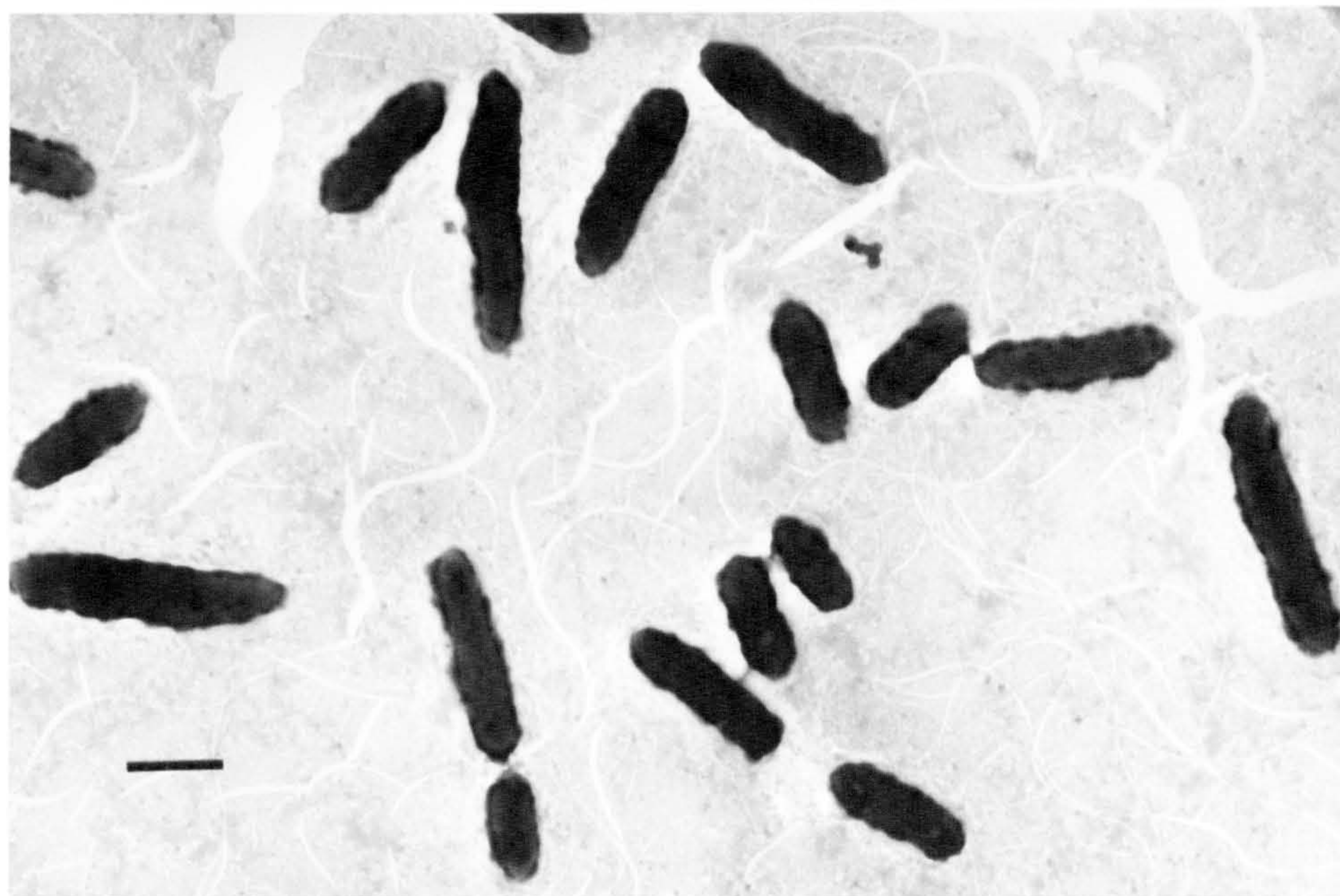


Fig. 20 The obligately heterotrophic moderate thermophile (from Kingsbury) grown on yeast extract (0.2g.l^{-1}). Bar marker, $1\text{ }\mu\text{m}$.

Fig. 21 Electrophoretic protein patterns from whole cell lysates of T. ferrooxidans (A), the Evenwood (B), Lake Myvam (C), Kingsbury (D) and Birch Coppice (E) isolates, thermophile TH1 (F) and the Alvecote isolate (G). All cultures grown on iron plus yeast extract.

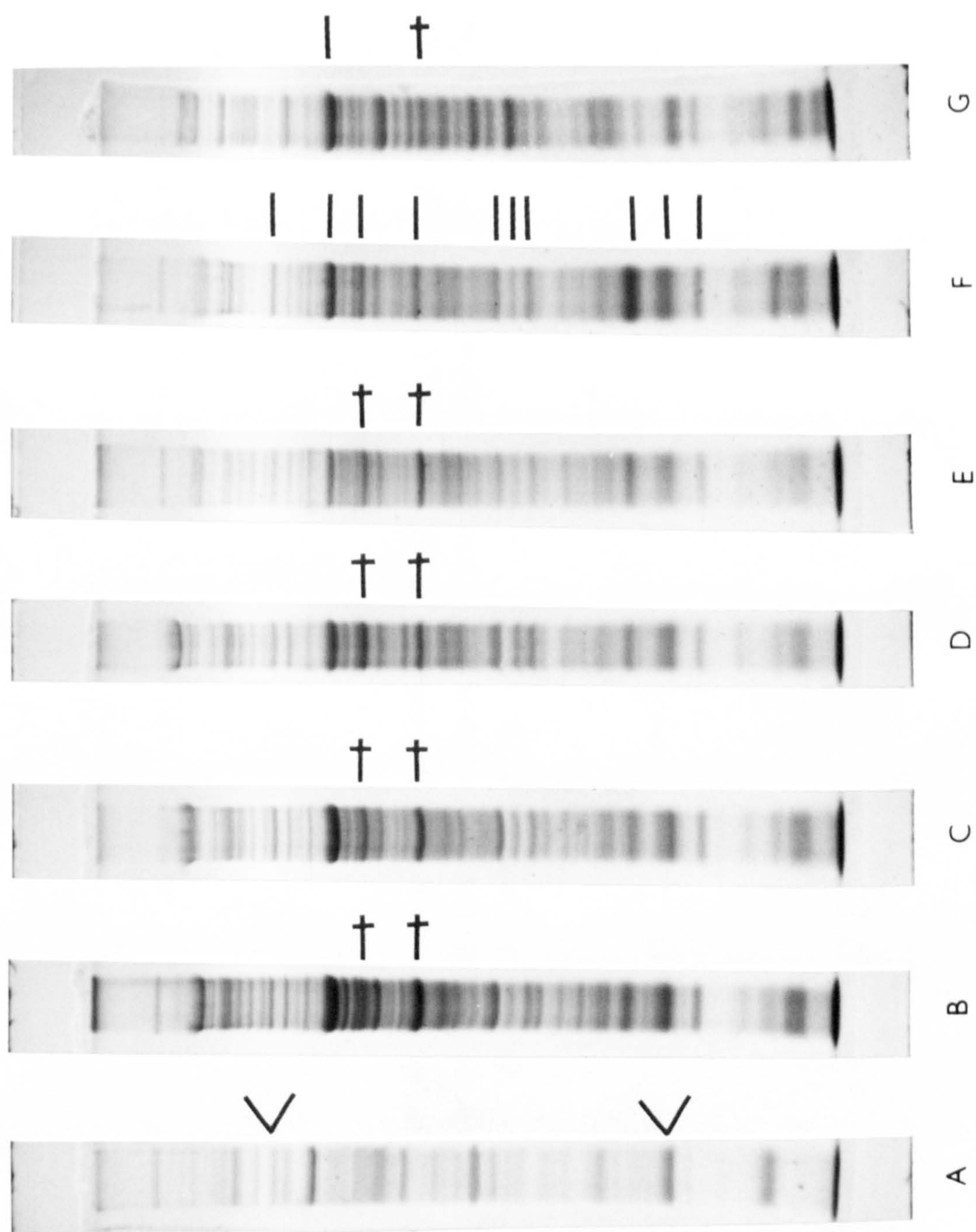
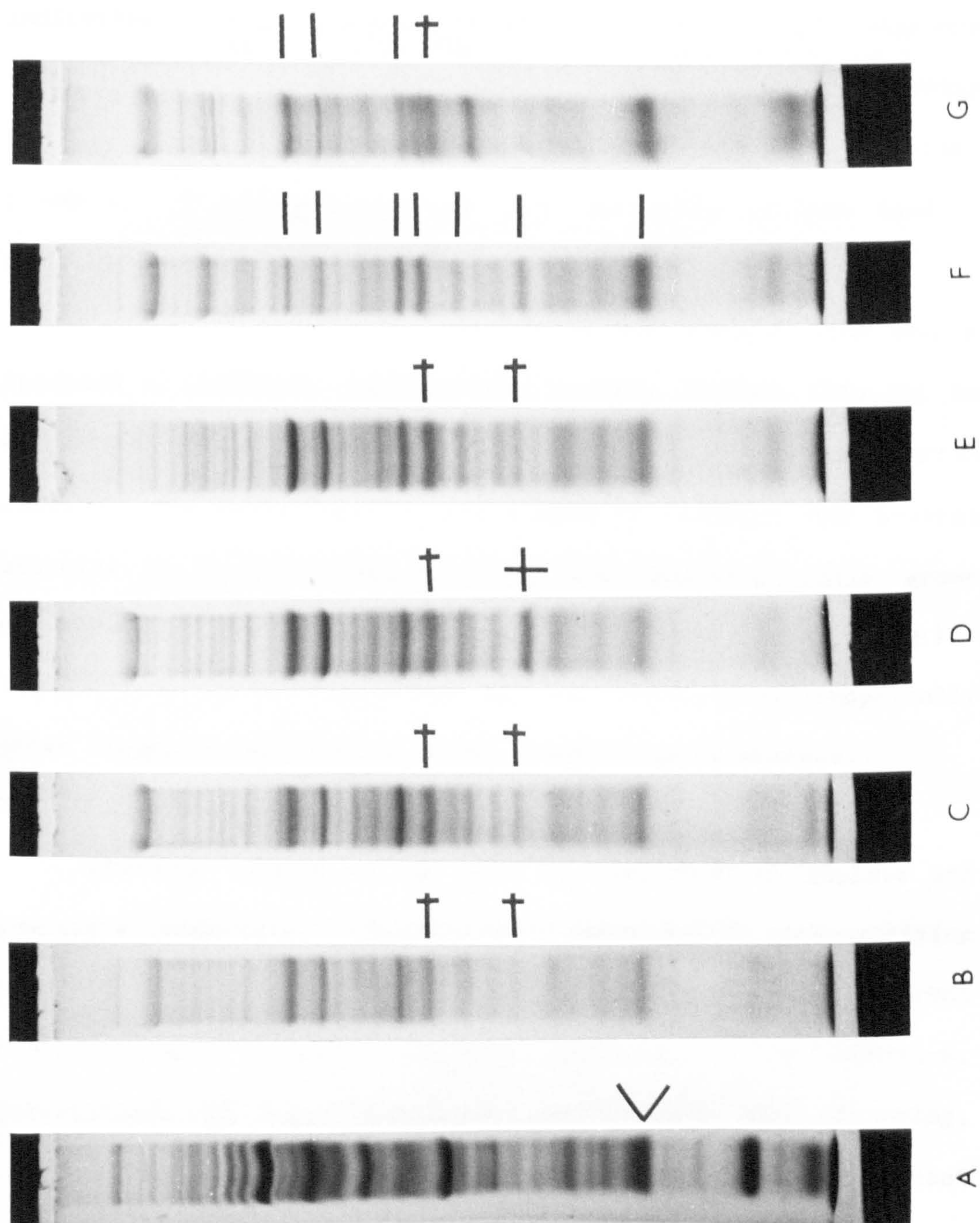


Fig. 22 Electrophoretic protein patterns from whole cell lysates of T. ferrooxidans (A), the Evenwood (B), Lake Myvam (C), Kingsbury (D) and Birch Coppice (E) isolates, thermophile TH1 (F) and the Alvecote isolate (G). All cultures grown autotrophically on iron.



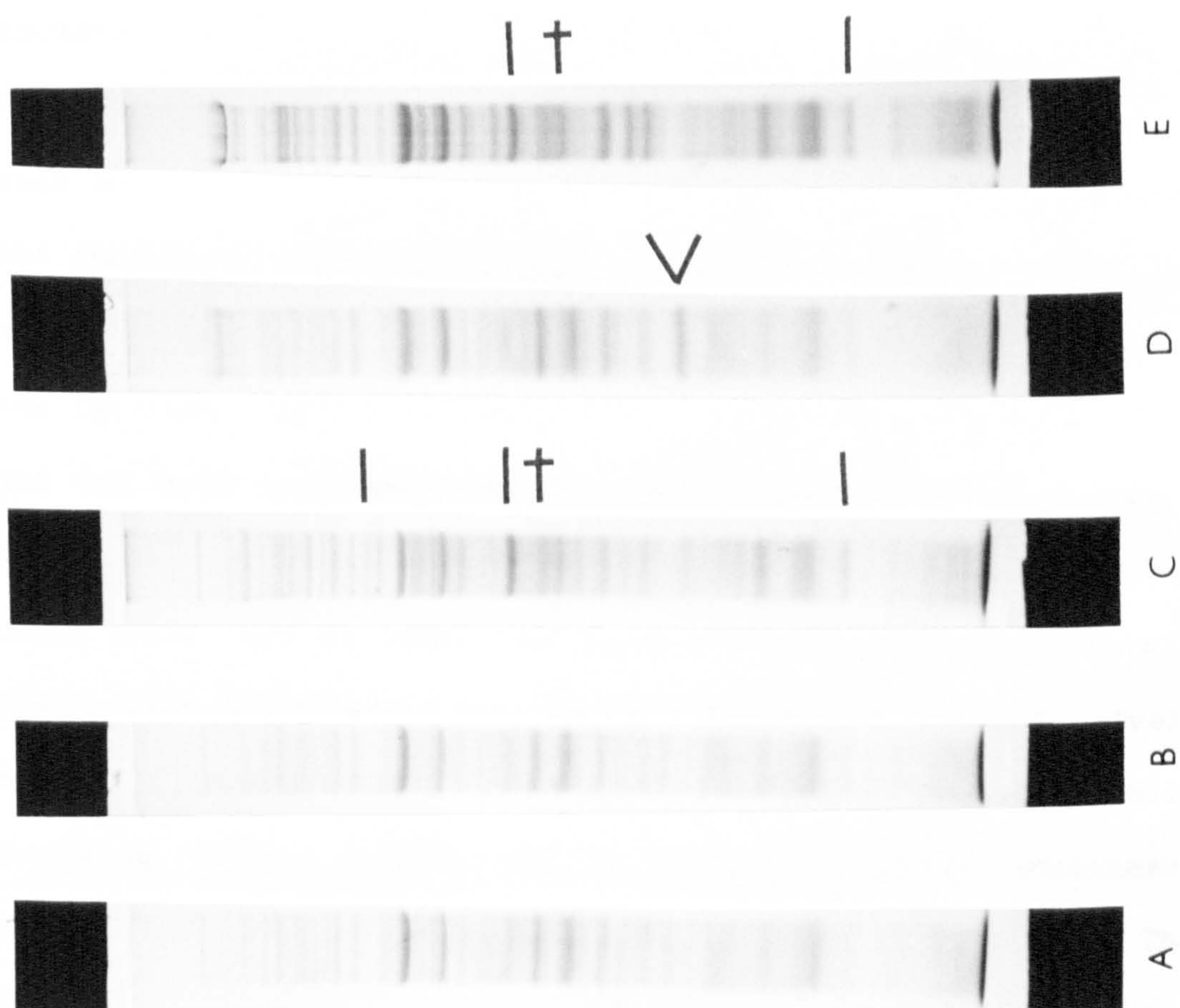
for comparison. With the Evenwood, Lake Myvam and Birch Coppice isolates, 5 of the bands were in identical positions to TH1 bands and perhaps 2 were slightly different (—+). The Kingsbury isolate had only one different major protein band to TH1 (—+) but one of the bands (+) in common with TH1 was very dark indicating a large quantity of protein present. The Alvecote isolate produced a protein band pattern with 3 bands being identical to TH1 (—) and one identical to the other isolates (—+). T ferrooxidans had only one major protein band position (<) in common with TH1 and the other isolates.

The presence of yeast extract in the ferrous iron medium produced a different cell-protein banding pattern (Fig.23) to autotrophically grown cells. Chemolithoheterotrophically grown cells of the Birch Coppice and Kingsbury isolates had several proteins in higher concentrations than autotrophically grown cells (—) and the positions of other bands had altered (—+). The presence of the heavy band of the chemolithoautotrophically grown Kingsbury isolate (<) was always clearly visible.

3.8 DISCUSSION

Although no attempt was made in this work to isolate and compare a large number of moderately thermophilic iron-oxidizing bacteria the isolates obtained showed some different electrophoresis protein banding patterns. In addition, descriptions of other organisms, particularly TH3 (Brierley, 1978) also suggest that many types probably exist. At least

Fig. 23 Electrophoretic protein patterns from whole cell lysates of the Birch Coppice isolate grown autotrophically (A and B) and in the presence of yeast extract (C) on iron and the Kingsbury isolate grown autotrophically (D) and in the presence of yeast extract (E) on iron.



four groups are now evident: the TH1 and Birch Coppice-like organisms; the Alvecote isolate; TH3 and LM2 (see Section 5 for description of this organism which has a different protein banding pattern and response to elemental sulphur compared with the TH1-like Lake Myvam isolate (LM1) described above).

The isolation of moderately thermophilic iron-oxidizing bacteria from coal mine sites has increased the habitat range of these organisms. There is obviously a widespread distribution of such bacteria with perhaps some strain variation occurring due to the specific, local conditions. It is also clear that there are mixed populations of iron-oxidizers at most sites, for example the TH1-like organisms designated TH2 (Brierley & Lockwood, 1977) and TH3 both originated from the same copper leaching site. Similarly, two types were isolated from the Lake Myvam sample (as noted above) and at least two types were present at Alvecote. The isolate described in this section was unable to progressively degrade pyrite in batch culture (Marsh & Norris, 1983) while extensive pyrite degradation by a 50°C Alvecote enrichment culture containing TH1-like organisms is noted in Section 7. The extent to which each type of thermophile is distributed geographically is unknown with, for example, the readily recognisable TH3 only observed so far in samples from the Chino leach dump.

The autotrophic growth of iron-oxidizing moderate thermophiles had not previously been demonstrated but by

providing cultures at all stages of enrichment and purification with a CO₂-enriched atmosphere and a source of reduced sulphur their autotrophic capacity was readily demonstrated. TH1 showed no loss of an autotrophic capacity despite being maintained over several years in the presence of yeast extract. Similarly, there was no loss of iron-oxidizing ability after repeated sub-culture on yeast extract alone.

The fastest autotrophic rates of iron oxidation, as illustrated by the Birch Coppice isolate, were similar to that obtained with T. ferrooxidans at 30°C (Norris & Kelly, 1982). This is surprising in that an increase in temperature might be expected to increase the rate of reaction provided that it was occurring at the optimum temperature for growth of the organism. It suggests that there are other growth factors which are limiting the autotrophic growth rates. Inhibition by ferric iron has been shown to reduce the rate of iron oxidation by T.ferrooxidans (Kelly et al., 1977). Another factor could be the nature of the reduced sulphur requirement. However, increasing the concentration of reduced sulphur in a batch culture only altered the duration of the faster rate and not the rate itself which suggests that the reduced sulphur was not limiting the rate of reaction. After the work described in this Section was completed, further study of the Alvecote thermophile described mixotrophic growth of the organism in ferrous iron medium supplemented with glucose but no source of reduced sulphur (Wood

& Kelly, 1984) and the isolate has also been grown autotrophically on iron in the absence of reduced sulphur (P.Norris, personal communication). Therefore, it seems that one strain can assimilate sulphate for biosynthesis but the other available strains all require reduced sulphur even when growing mixotrophically in the presence of glucose. The capacity to use an inorganic source of reduced sulphur during autotrophic growth on iron by the moderate thermophiles therefore remains a significant factor when considering the potential industrial use of such organisms. However, in most environments where these bacteria are active it is probable that there would be sufficient reduced sulphur to support autotrophic growth on iron.

The presence of heterotrophic bacteria has been described in all the environments from which the moderate thermophiles have been isolated (Ehrlich, 1963; Harrison, 1978; Wichlacz & Unz, 1981) but little is known of their effect on the iron oxidation rate by the thermophiles. It is evident that the environmental pH has an effect on the growth of such heterotrophs. In situ at pH values near 2.0 it is possible that the heterotrophs would grow on the available organic matter and the iron-oxidizers might therefore need to adopt an autotrophic mode of growth on ferrous iron with a resulting reduction in their growth rate.

Mixotrophic growth would be the other available option and this has been demonstrated with a mixture of glucose and CO₂ by the Alvecote, Birch Coppice and Kingsbury isolates (Wood & Kelly,

1983) although no dramatic increase in yields over autotrophic growth occurred.

The ability of the moderately thermophilic iron oxidizers to grow at temperatures above their optimum has been shown to be dependent on inoculum size and external pH. TH1 would only grow at 60°C if the initial pH was a maximum of 1.6 and a large inoculum (5-10% v/v) was used. The occurrence of an enrichment culture from Kingsbury which could readily oxidize iron at 60°C and showed an optimum temperature of 55°-60°C extends the temperature range over which the moderate thermophiles have been reported to grow. The maximum temperature for iron oxidation by T. ferrooxidans increases as the pH increases from 1.5 to 2.0 (P.Norris, unpublished data). This did not occur with the moderate thermophiles which showed a poorer rate of iron oxidation at the higher pH values which could be due to the extensive precipitation coating the surface of the bacteria.

The electrophoregrams of cell protein lysates from the moderately thermophilic bacteria confirm the physiological data in that they clearly belong to a different genus to T.ferrooxidans. Yeast extract has little effect on the iron oxidation rate of T. ferrooxidans as it grows autotrophically on ferrous iron even in its presence. If the Kingsbury isolate was similar in this respect the prominent protein band occurring in the chemolithoautotrophically grown cells could indicate the presence of a carboxylase enzyme, fixing CO₂, in larger

quantities than in the other isolates. The autotrophic yields of the Kingsbury and Alvecote isolates have been shown to be similar to T. ferrooxidans and higher than that of the Birch Coppice isolate (Wood & Kelly, 1983). This variation in the ability of the isolates to grow autotrophically on ferrous iron is another example of the variations that exist in the moderately thermophilic bacteria.

Comparing the isolates by physiological methods and electrophoregrams does show the similarities between the organisms and are useful procedures in classification. However, more detailed analysis is required to ascertain the degree of similarity to one another. Visual examination of electrophoregrams gives a useful guide to organism interrelationships but densitometer tracings of the protein patterns against reference proteins would produce a more critical and objective analysis of results (Kerstens & DeLey, 1975; 1980). DNA-DNA homologies have shown that mesophilic strains previously designated as T. ferrooxidans fall into at least 7 homology groups, none of which show any genomic affinity with strains of T. thiooxidans (Harrison, 1982). Such a technique would be useful in classifying the moderately thermophilic iron-oxidizing isolates and determining the number of species and strains isolated.

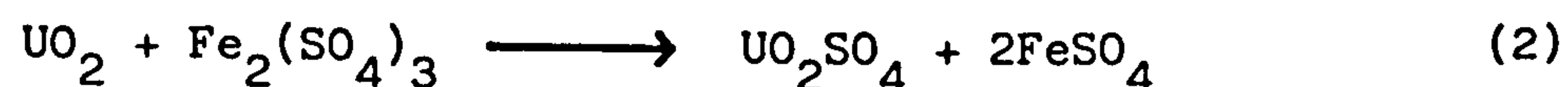
P A R T 4

BACTERIAL FILM CONTINUOUS IRON OXIDATION

4.1 INTRODUCTION

During batch and continuous oxidation of ferrous iron by the mesophile T. ferrooxidans the presence of a bacterial film has been observed on the culture vessel surfaces (MacDonald & Clark, 1970). The ferric sulphate formed by the oxidation process is hydrolysed to form basic ferric sulphates called jarosites of the type $MFe_3(SO_4)_2(OH)_6$ (where M is Na^+ , K^+ , NH_4^+ or H^+) and hydrated ferric oxides (Brierley, 1978; Lazaroff et al., 1982). T. ferrooxidans attaches to or is incorporated in this precipitate to form a bacterial film which has a greater effect on the rate of iron oxidation than the free bacteria in suspension (MacDonald & Clark, 1970; Groudev, 1981; Mehta & LeRoux, 1974). Washout from a continuous oxidation vessel can be avoided provided the film remains moist and the vessel can be operated at higher throughput rates than a vessel where no bacterial film is present (Mehta & LeRoux, 1974).

Acidified ferric sulphate solutions can be used to oxidize sulphide minerals to their respective sulphates (equation 1, where M is a bivalent metal) and to solubilize uranium (iv)



to uranium (vi) (equation 2). It is therefore necessary in some leaching operations to oxidize and recycle the ferrous iron formed. This can be done by chemical processes but can also be achieved by bacterial oxidation. The use of a bacterial film of T. ferrooxidans has been employed in an industrial process at the

Buffelsfontein Uranium Plant, South Africa, with a process known as BACterial Film OXidation (BACFOX).

Prior to the use of bacteria the uranium ore was milled to 70% minus 0.074 mm and hot leached at 50°C to ensure maximum extraction with dilute H_2SO_4 /ferric sulphate liquor (Livesey-Goldblatt et al., 1977). The ferrous iron was oxidized chemically by the addition of manganese dioxide.

Trials with several types of iron-oxidizing plant found that the corrugated packing, forced aeration type, gave the highest specific ferrous iron oxidation rate when measured as grams of iron oxidized per square metre per hour ($\text{g.m}^{-2}\text{h}^{-1}$) (Livesey-Goldblatt et al., 1977). The reaction vessel contained 1,080 l with a surface area including packing material of 100m^2 . Aeration from the bottom of the tank was at the rate of $50\text{ m}^3\text{h}^{-1}$. The corrugated packing was present to provide an increased surface area for a bacterial film to develop. When this was scaled up into a rubber-lined tank 12' high by 50' diameter the corrugated packing was replaced by 2" diameter plastic pipes which could be more easily cleaned when there was an extensive build up of precipitates (E. Livesey-Goldblatt, personal communication).

The pilot plant trials showed that continuous oxidation of ferrous iron occurred when the intake contained between 3.5-15.0 $\text{g.Fe}^{2+}\text{l}^{-1}$. At approximately $5\text{g Fe}^{2+}\text{l}^{-1}$ a maximum specific rate of oxidation of $7.5\text{g.m}^{-2}\text{h}^{-1}$ was achieved after 45 days. The commercial plant was run at pH 2.0 with an intake containing

5.5-6gFe l^{-1} , mainly in the ferrous form, and took approximately 10 weeks to reach maximum oxidation rates.

The available water at a site where oxidation and recycle of ferrous iron might be desirable may not be suitable for supporting growth of iron-oxidizing bacteria. For example, the presence of high chloride ion concentrations could be inhibitory to bacterial growth. At the Uranium deposit at Honeymoon, Australia, the only available water which could have been used for ferric iron circulation in a leaching operation was highly saline (8.4gCl l^{-1}). Levels of chloride as high as this have been shown to inhibit iron oxidation by T. ferrooxidans (Tuovinen & Kelly, 1972). Inhibition of iron oxidation has been shown to occur at 3gCl l^{-1} (Tuovinen et al., 1971b) and although growth has been demonstrated at 6.0-7.0gCl l^{-1} prolonged exposure to 7.0gCl l^{-1} produced severe inhibition (P. Norris, personal communication; Tuovinen et al., 1971). However, different strains of T. ferrooxidans have shown a range of sensitivity to the presence of chloride ions (P. Norris, personal communication). Initial work in batch culture using the moderate thermophile TH1 indicated that the rate of iron oxidation was unaffected by levels of 8.0gCl l^{-1} , although it produced an extension in the lag phase of growth (D.P. Kelly, personal communication). Different moderate thermophiles showed different tolerance levels with iron oxidation by TH3 being less inhibited by Cl $^{-}$ when tested over the range 2.8-28.2mM during measurements of oxygen uptake by non-growing suspensions in an oxygen

electrode.

The aim of this work was to test if moderately thermophilic iron-oxidizing bacteria which have been shown to have a faster doubling time for iron oxidation than T.ferrooxidans in batch culture (Section 3) were capable of forming a bacterial film and allowing a faster rate of specific iron oxidation. The maximum potential rates per unit vessel volume would be unlikely to be achieved due to limitations in the reaction vessel design but a comparison could be made between continuous iron oxidation by T. ferrooxidans and a moderately thermophilic bacterium. Once steady-state continuous rates of iron-oxidation with different organisms were achieved the system could be stressed by increasing chloride additions to test the relative tolerances of the different bacteria.

4.2 THE EFFECT OF TUBING COMPOSITION ON IRON OXIDATION BY T.FERROOXIDANS AND MODERATELY THERMOPHILIC BACTERIA

During initial runs on iron oxidation in the vessels active cultures of T. ferrooxidans continued to grow when the switch from batch to continuous culture was made but the Evenwood moderate thermophile culture was washed out of the pot in a few days. Microscopic observations showed that the Evenwood bacteria had a distorted appearance. During these runs, silicone rubber tubing was used on all the medium inlet lines and it was suspected that the tubing could have been the source of toxic compounds.

10 cm lengths of a range of tubing types were left in the

ferrous iron medium supplemented with yeast extract at room temperature for 125 hours before being removed. The flasks were then inoculated with thermophile TH1 and incubated at 50°C. Growth occurred in the flasks that had previously contained PVC, PTFE, vinyl and polythene tubing (Fig.24) but not in the flasks that had contained silicone rubber, rubber or neoprene tubing. Following the same procedure subsequent iron oxidation by T.ferrooxidans at 30°C was unaffected by the tubing-medium contact. The tubing which did not have a toxic effect on growth of the moderate thermophile was not suitable for use in a Watson-Marlow peristaltic pump as it was either too rigid (PVC, PTFE and polythene) or insufficiently durable to be used continuously (vinyl). For this reason the inlet medium lines were constructed of glass tubing with PVC or PTFE being used as connectors. A short piece of narrow-bore silicone tubing (1mm diameter) was used through the pump.

4.3 COMPARISON OF CONTINUOUS FERROUS IRON OXIDATION BY T.FERROOXIDANS AND THE EVENWOOD MODERATE THERMOPHILE

Iron oxidation in vessel 1 containing T. ferrooxidans without additional support material (Fig.25) reached 95% oxidation before the flow was started at 15 ml h⁻¹. At 175h and 325h the flow rate increased through pump malfunction resulting in a fall in the level of iron oxidation in the vessel. The flow was therefore switched off and the iron oxidation level allowed to reach 60% before the flow was restarted. The amount of ferric iron produced and the conversion rate gradually increased so that

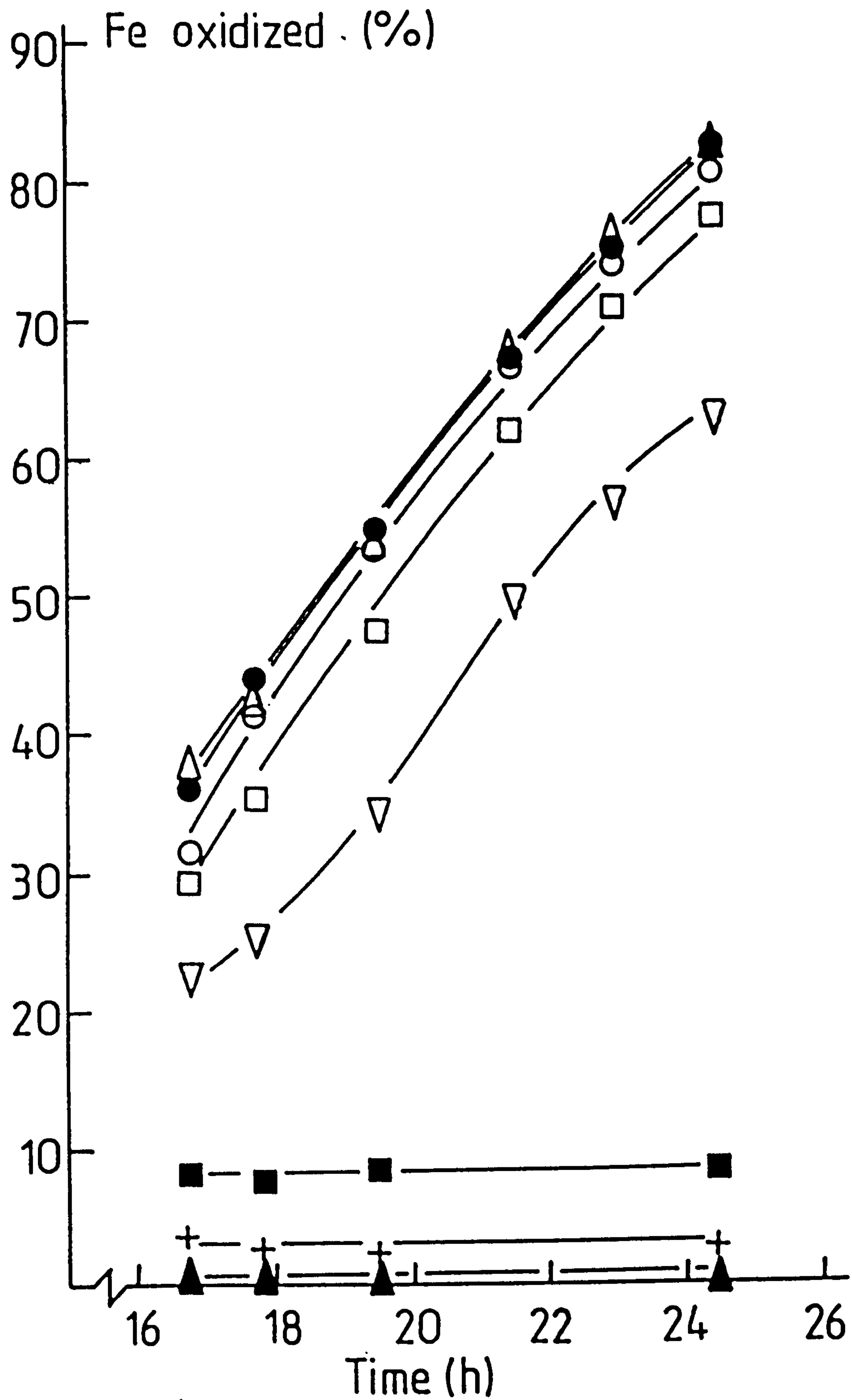
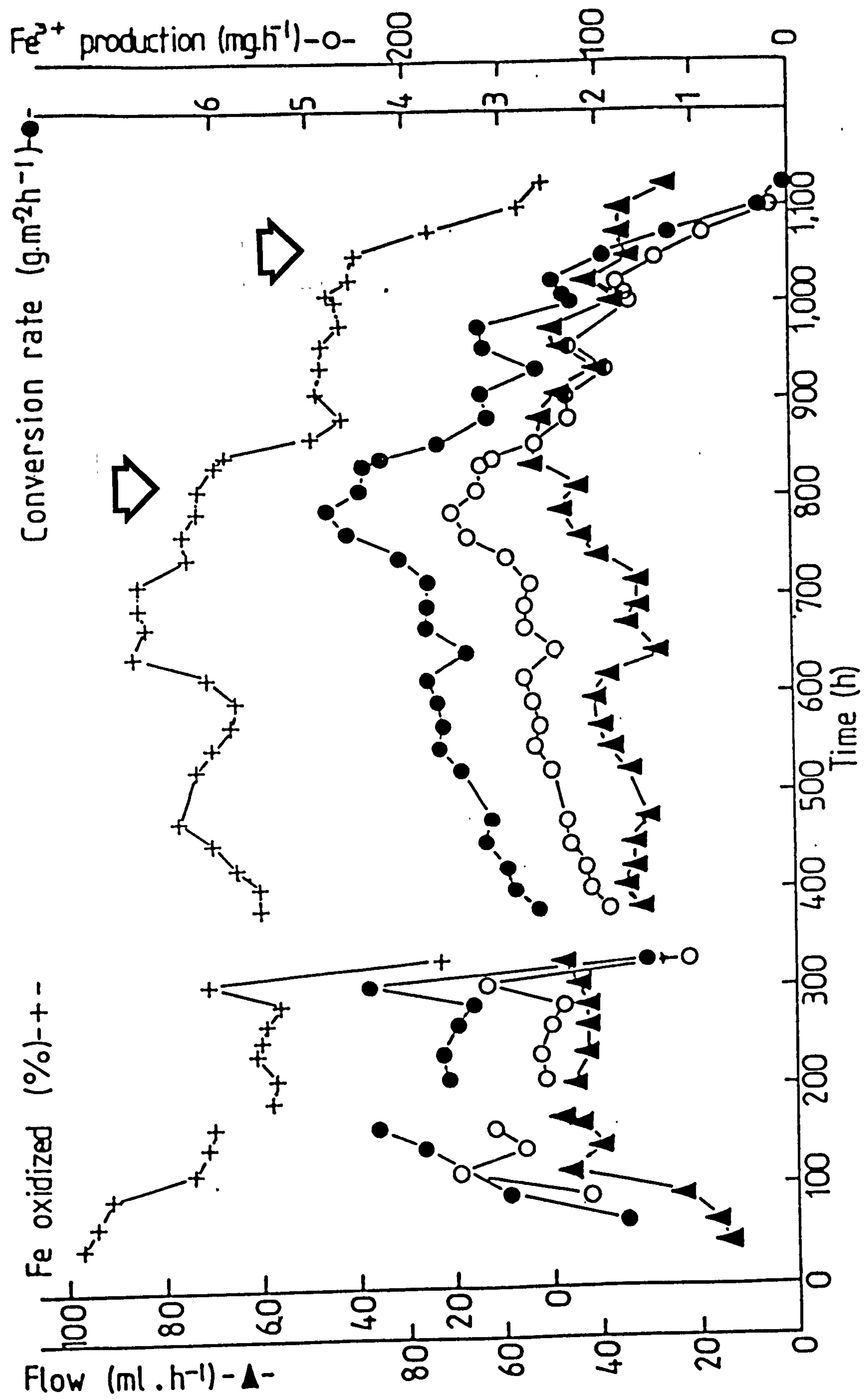


Fig. 24 The effect of tubing constituents on growth of thermophile TH1 at 50°C. Ferrous iron oxidation in the standard medium (●) and in medium previously in contact with silicone (+), rubber (▲), neoprene (■), PVC (▽), PTFE (○), vinyl (□) and polythene (△) tubing.

Fig. 25 Iron oxidation by T. ferrooxidans at 30°C in a continuous flow vessel. Medium flow rate, percentage iron oxidized and ferric iron production rates are shown.

Arrows indicate additions of chloride (see page 91)



by 775h, at 70% oxidation, $160 \pm 10\text{mg Fe}^{3+}\text{h}^{-1}$ were being produced with a conversion rate of $4.5 \pm 0.2\text{g.m}^{-2}\text{h}^{-1}$.

Iron oxidation in vessel 2 containing T. ferrooxidans with the additional support material (Fig.26) reached 95% oxidation before the flow was started at 15 ml h^{-1} . The flow rate was gradually increased and by 350h at 70% oxidation, $250 \pm 20\text{mgFe}^{3+}\text{h}^{-1}$ were being produced with a conversion rate of $2.0 \pm 0.2\text{g.m}^{-2}\text{h}^{-1}$. This rate of ferric iron production was reached more quickly than in vessel 1. Increasing the flow rate from 90 to 115 ml h^{-1} (600h) resulted in a decrease in % oxidation. Decreasing the flow rate to 65 ml h^{-1} (800h) caused an increase in % oxidation to $85 \pm 5\%$, an increase in ferric iron production to $350 \pm 10\text{mg h}^{-1}$ and an increase in conversion rate to $2.5\text{g.m}^{-2}\text{h}^{-1}$. However, these higher rates were not maintained when the flow rate was returned to 90 ml h^{-1} .

Vessel 3 containing the Evenwood moderate thermophile (Fig. 27) reached 95% oxidation before the flow was started at 10 ml h^{-1} . After 350h, at approximately 65% oxidation, the rate of Fe^{3+} production was $170 \pm 10\text{mg h}^{-1}$ with a conversion rate of $4.4 \pm 0.4\text{g.m}^{-2}\text{h}^{-1}$. The sudden fall in Fe^{3+} production and conversion rate at 200h and 650h was caused by a reduction in the flow rate.

Vessel 4 containing the Evenwood moderate thermophile with additional support material (Fig.28) reached 95% oxidation before the flow was started at 10 ml h^{-1} . After 400h, at approximately 60% oxidation, the Fe^{3+} production rate was $275 \pm 25\text{mg h}^{-1}$ and the conversion rate $2.0 \pm 0.25\text{g.m}^{-2}\text{h}^{-1}$. At 200h there was a

Fig. 26 Iron oxidation by T. ferrooxidans at 30°C in a continuous flow vessel with an increased internal surface area.

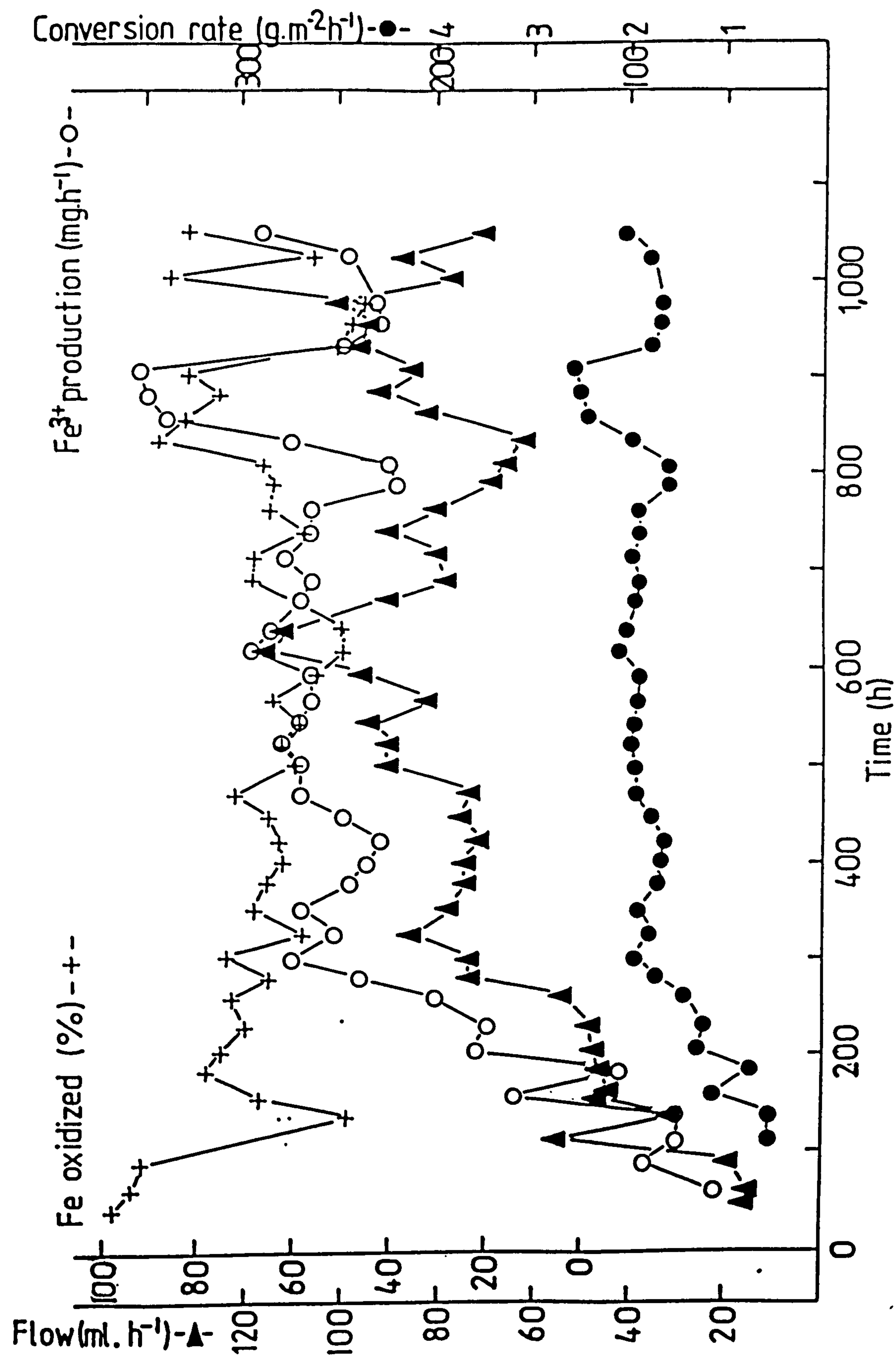


Fig. 27 Iron oxidation by the Evenwood moderate thermophile at 50°C in a continuous flow vessel. The medium was supplemented with yeast extract (0.2g.l^{-1}). The added chloride concentration is shown as well as the iron oxidation and ferric iron production rates.

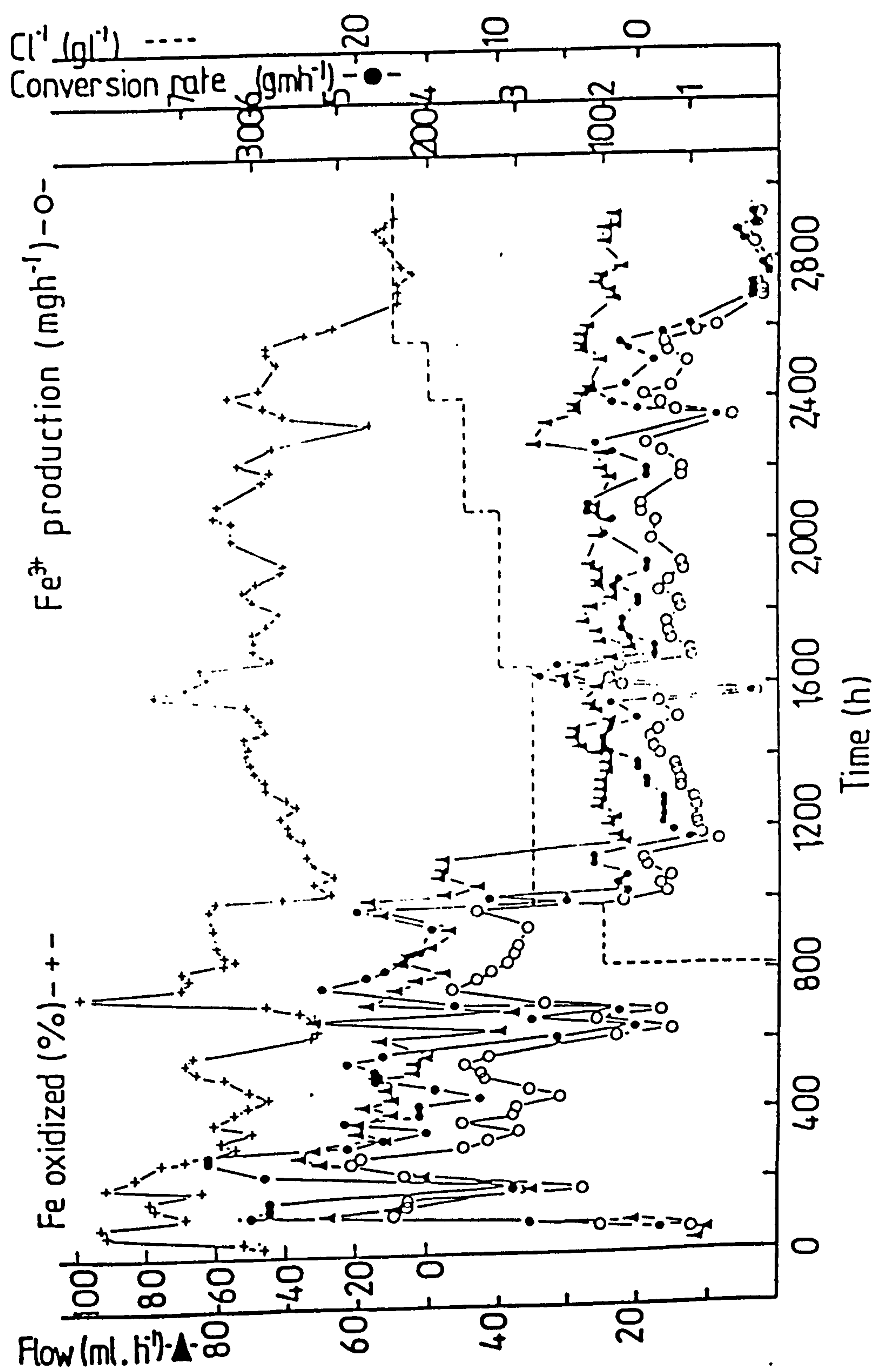
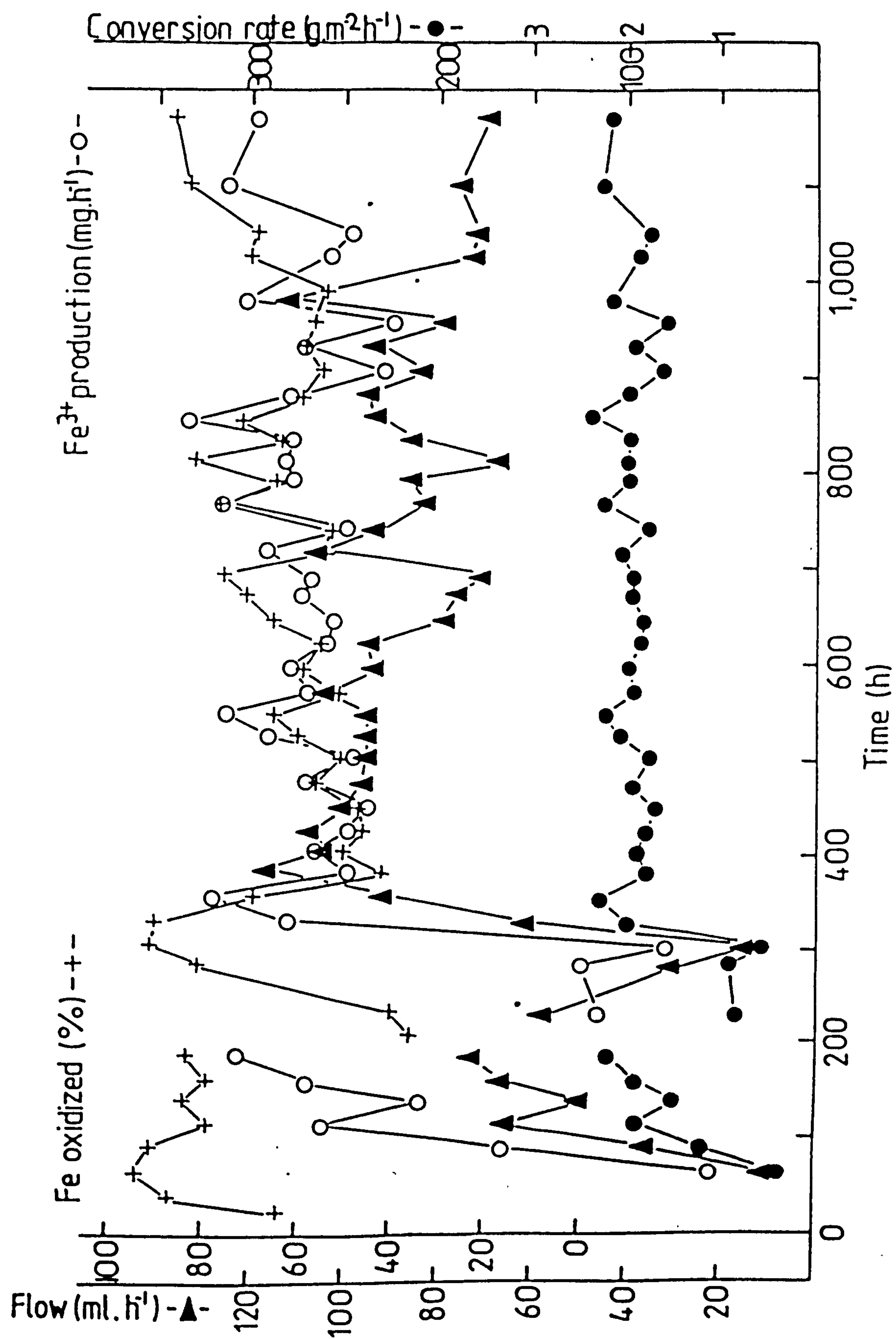


Fig. 28 Iron oxidation by the Evenwood moderate thermophile at 50°C in a continuous flow vessel with an increased internal surface area. The medium was supplemented initially with 0.2g yeast extract l^{-1} and with 0.4g yeast extract l^{-1} after 690h operation.



sudden drop in the percentage of oxidized iron in the vessel from 85 to 35% and the flow was stopped. At 690h the level of yeast extract was doubled to 400mg l^{-1} but this did not produce a significant improvement.

A photograph of vessels 2 and 4 after the experiment was concluded (Fig.29) shows the difference in the extent of precipitation on the glass slides. At 50°C the precipitate was thick and uneven causing a reduction in liquid volume from 220 to 180 ml during the 1, 170 hours the vessel was run. At 30°C the precipitate was thinner with a smoother surface so that the final liquid content of the vessel was only reduced to 210 ml.

4.4 THE EFFECT OF CHLORIDE IONS ON THE CONTINUOUS OXIDATION OF FERROUS IRON BY T. FERROOXIDANS AND THE EVENWOOD MODERATE THERMOPHILE

Adding $2.5\text{g Cl}^{-1}\text{l}^{-1}$ to T. ferrooxidans (vessel 1, Fig.25) at 815h resulted in a gradual decrease in percentage oxidation. By 900h this had stabilized at 45% but the Fe^{3+} production rate had fallen by 50mg h^{-1} and the conversion rate by $1.25\text{gm}^{-2}\text{h}^{-1}$. These rates continued to decrease indicating that there was no apparent recovery. When $7.5\text{g Cl}^{-1}\text{l}^{-1}$ was added at 1,055h the vessel washed-out even at low flow rates.

Adding $2.5\text{g Cl}^{-1}\text{l}^{-1}$ to the Evenwood moderate thermophile (vessel 3, Fig.27) produced a fall of 40mg h^{-1} in the Fe^{3+} production rate over the same 240h period. When the level of Cl^{-} was increased to 7.5gl^{-1} there was a marked decrease in Fe^{3+} production rate from 170 to 60mg h^{-1} with oxidation still

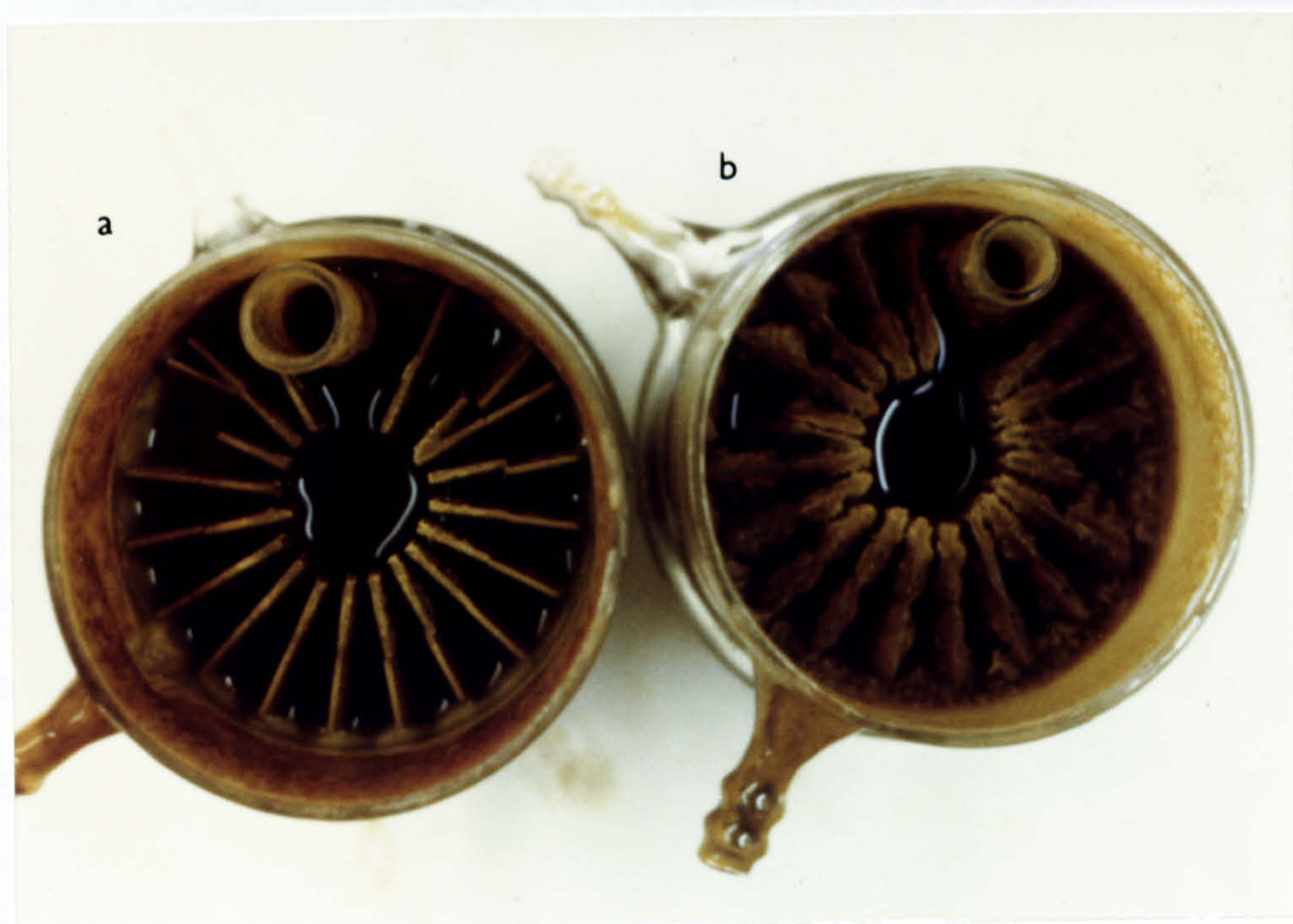


Fig. 29 The continuous flow vessels with increased surface areas at the completion of the experiments at (a) 30°C and (b) 50°C.

occurring at $40 \pm 10\%$. The rate of iron oxidation, Fe^{3+} production and conversion rate then remained relatively stable despite an increase in Cl^- until at 2,570h a level of $17.5\text{gCl}^- \text{l}^{-1}$ was reached. At this point there was a fall in the level of iron oxidation to 10% and a corresponding decline in Fe^{3+} production level to 10mg h^{-1} , at which point the experiment was concluded. Microscopic examination of the cells showed them to have a short, distorted appearance.

4.5 DISCUSSION

The softer types of tubing (silicone, rubber and neoprene) which inhibited growth of the moderate thermophiles contain plasticizers and it has been shown that these substances can be toxic to the growth of microorganisms (Timms, 1981). These substances must have dissolved to the same extent in the medium used for both T. ferrooxidans and the moderate thermophile because all the inlet medium was at room temperature as in the tubing toxicity experiment. Although the initial pH of the medium was different for the two organisms (T. ferrooxidans, pH 2.0; moderate thermophile, pH 1.6) in the continuous iron oxidation vessels, in the tubing experiment the initial pH of the medium was the same for both organisms (pH 1.7). Therefore the more acid pH of the inlet medium for the moderate thermophile, which was required to avoid excessive precipitation and reduction of iron oxidizing ability (Section 3.4), was not a major factor in causing the toxicity of the tubing to the moderate thermophiles. Either growth of T. ferrooxidans was unaffected by

the plasticizers while growth of the moderate thermophiles was inhibited or at 50°C the nature of the plasticizers in the medium changed, making them toxic. The short piece of silicon tubing used through the pump did not noticeably inhibit growth but ideally, a pumping system which avoided the use of any silicon tubing would have to be used to eliminate any possible toxic effects.

When T. ferrooxidans was used to form a bacterial film, increasing the surface area 3.8 times produced a corresponding two-fold increase in Fe^{3+} production. However, although the Fe^{3+} production per hour had doubled the specific rate per unit area was halved as the surface area was increased almost four-fold. This lower than expected value indicates that either an efficient bacterial film had not developed perhaps due to an inadequate oxygen supply or that the small nature of the reaction vessel was allowing large amounts of ferrous iron to leave the vessel before being oxidized. The maximum specific rate of oxidation of Fe^{2+} to Fe^{3+} achieved by the pilot-plant units of the BACFOX process was $7.5\text{gm}^{-2}\text{h}^{-1}$ (Livesey-Goldblatt et al., 1977). The reaction vessels during this experiment reached only $2.5\text{gm}^{-2}\text{h}^{-1}$ but this is probably due to the limitations of the vessel and as these constraints were the same in all vessels comparisons between them were still able to be made.

The Evenwood moderate thermophile showed a similar rate of ferric iron production to that of T. ferrooxidans; not a faster rate as would have been expected from batch culture with chemolitho-

heterotrophic growth (see Section 3.4). The yeast extract was not a limiting factor as there was no improvement in iron oxidation rate as would otherwise have been expected with a doubling of the yeast extract concentration. The extensive precipitation in the 50°C vessel could have prevented an efficient bacterial film forming as attached bacteria would be continually covered with a fresh layer of precipitate so preventing further oxidation. Attempts were made to look at the development of a possible bacterial film using fluorescence microscopy but the build up of precipitate prevented any reliable estimate of bacterial numbers from being made. The vessel at 50°C could be run at a lower pH, e.g. 1.5, which would decrease the extent of the precipitation and an organism adapted to grow optimally at this pH could be used. Autotrophic cultures of moderate thermophiles, which were demonstrated after these experiments were done, could be tried under conditions of continuous iron oxidation but they could well not show any faster rates as batch cultures have a doubling time for iron oxidation similar to that of T. ferrooxidans (see Section 3.4).

As relatively poor bacterial film oxidation occurred in the vessels with both T. ferrooxidans and the moderate thermophile, consideration could be given to Leptospirillum ferrooxidans. Clumps of cells are visible in iron-grown batch cultures of L.ferrooxidans which suggests that they could be secreting an external polymer which allows the cells to attach to each other. A true biofilm could possibly develop, under the right conditions

allowing a dense film of bacteria to form and a correspondingly higher rate of specific iron oxidation than with T.ferrooxidans or the moderate thermophile where the bacteria are only attached to the precipitate and not one another.

However, one advantage of using the moderately thermophilic bacteria is their higher tolerance of Cl^- with the Evenwood moderate thermophile showing severe inhibition of iron oxidation at $17.5\text{gCl}^- \text{l}^{-1}$ compared to $7.5\text{gCl}^- \text{l}^{-1}$ for T. ferrooxidans. The moderate thermophiles could therefore be used in areas where the only available water had a high chloride content. This tolerance level might be increased by using different strains or selecting for higher chloride concentration-tolerant mutants.

P A R T 5

MODERATELY THERMOPHILIC SULPHUR-OXIDIZING BACTERIA

5.1 INTRODUCTION

Rod-shaped bacteria growing autotrophically on elemental sulphur up to a temperature of 55°C have been isolated from hot springs and soils around the world (Schwartz & Schwartz, 1965; Schoen & Ehrlich, 1968; Fliermans & Brock, 1972) and described as high temperature strains of Thiobacillus thiooxidans. They have frequently shown poor growth or failed to grow without a carbon dioxide enriched atmosphere (Fliermans & Brock, 1972). Such organisms are thought to contribute to the production of sulphuric acid in acidic, thermal habitats and occupy a temperature niche between that of the lower temperature T. thiooxidans strains and the extremely thermophilic Sulfolobus organisms. However, beyond the initial isolations of such organisms little work has been reported on any further characterization.

The iron-oxidizing moderate thermophile TH1 has been described as growing poorly on elemental sulphur in the presence of yeast extract (Brierley et al., 1978) with less growth being observed at each serial sub-culture. Oxygen uptake could not be detected with elemental sulphur in an oxygen electrode by a cell suspension of TH1 (Brierley et al., 1978).

This chapter describes further attempts to measure the growth on sulphur of the moderately thermophilic, iron-oxidizing TH group of organisms and reports the isolation of new sulphur-oxidizing strains. The growth, morphology and cell protein electrophoregrams of the new isolates were compared with a characterized strain of T. thiooxidans.

5.2 SELECTION OF A MEDIUM FOR THE ISOLATION OF ORGANISMS AND THE GROWTH STUDIES

The growth of thermophile TH1 on sulphur at 50°C in the presence of yeast extract (200 mg.l⁻¹) in five different salts media (see Section 2.4.3) was compared with the growth of T. ferrooxidans at 30°C in the same media. T. ferrooxidans was chosen in preference to T. thiooxidans so that organisms which had the capacity to oxidize both iron and sulphur were involved in the comparison. Growth by TH1 on sulphur was often difficult to establish over several sub-cultures. However when successive growth did occur the greatest fall in culture pH was observed using the low salt concentration medium of Allen and using the sulphur salts in which the pH fell from 3.1 to 1.5. (Fig.30a). Growth in the higher salt concentration media of Bounds and Colmer and 9K salts produced a smaller drop in culture pH from about 3.2 to 2.7. During growth of T. ferrooxidans (Fig.30b) the pH fell from about 3.0 to 1.2 in all the salts media. The lag phase before growth commenced was longer than with TH1 and in all media, except that of Hutchinson, a noticeably slower sulphur oxidation rate was observed. (The actual rates as measured by sulphate production were not determined). The sulphur salts medium was therefore chosen for the isolations and experiments as it seemed the most suitable for allowing a rapid assessment of growth, by pH measurement, of a moderate thermophile.

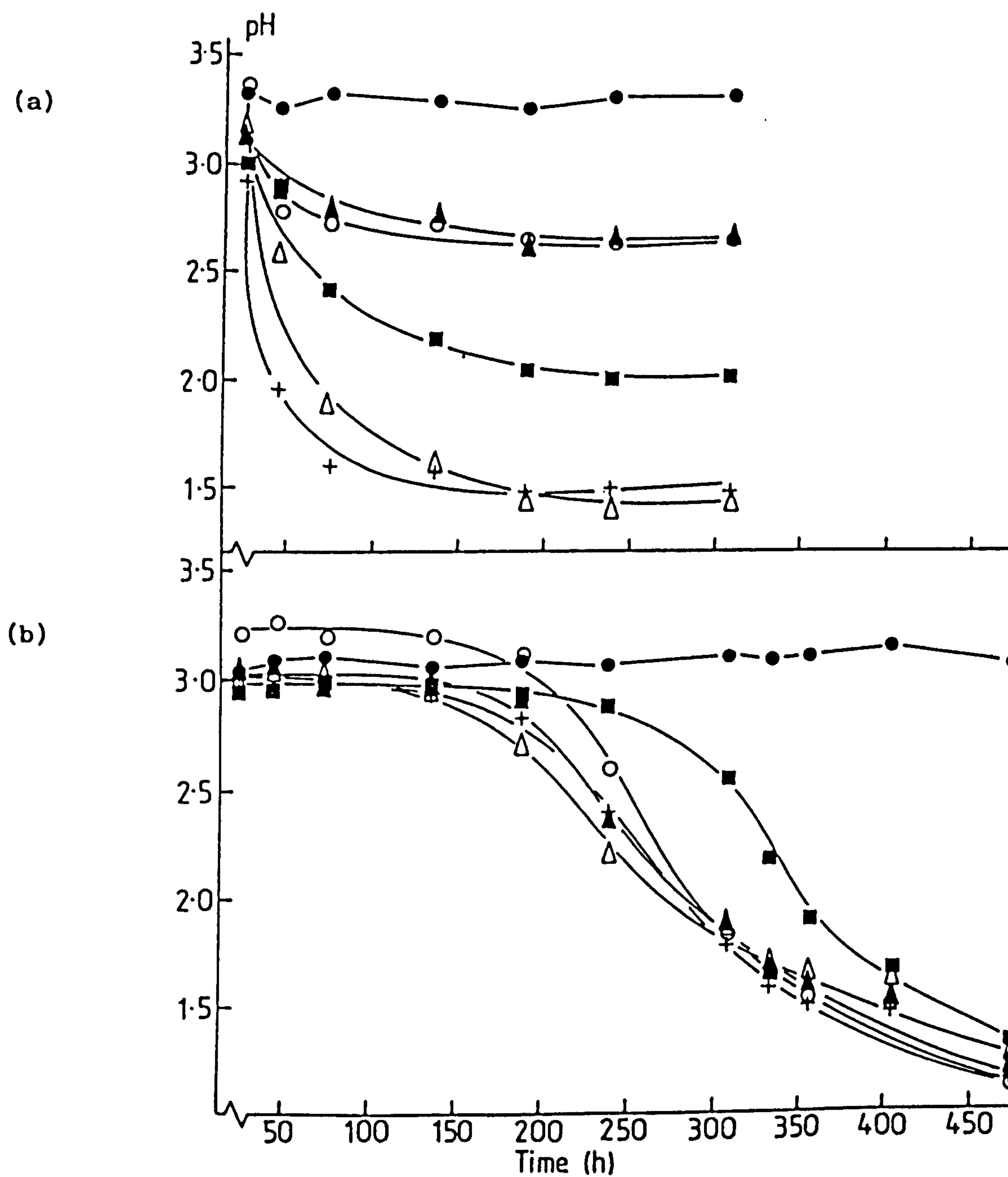


Fig. 30 The pH during growth on flowers of sulphur in the presence of yeast extract (0.2g.l^{-1}) by (a) TH1 and (b) *T. ferrooxidans* in the medium of Bound and Colmer (▲), Silverman and Lundgren (○), Hutchinson (■), Allen (+) and in the sulphur salts medium (Δ) and the pH in sterile controls (●).

5.3 ISOLATION OF SULPHUR-OXIDIZING BACTERIA AT 37°C AND 50°C

Organisms were isolated from the Birch Coppice and Lake Myvam samples via CO₂-enriched sulphur enrichment cultures at 50°C. The Birch Coppice isolates were purified via thiosulphate agar plates (see Section 2.7.2) but the Lake Myvam isolate growing at 50°C was only able to be grown on tetrathionate agar plates (see Section 2.7.2). Further work was carried out on isolates designated BC2, BC4 and LM2 as these appeared morphologically different under the microscope.

Organisms from CO₂-enriched sulphur enrichment cultures at 37°C were isolated and purified on thiosulphate agar plates from the Birch Coppice and Lake Myvam samples by P. Norris concurrently with the work at 50°C. Two of these isolates, designated BC13 and LM7, have been used for comparative purposes in this study.

Enrichment cultures on sulphur at 50°C from Kingsbury, Evenwood and Alvecote could only be maintained in the presence of yeast extract and were not purified or studied further.

Growth of sulphur-grown TH1 cultures could not be established on thiosulphate or tetrathionate agar plates. Cultures of TH1 were serially sub-cultured three times on sulphur before being used in experimental work.

5.4 GROWTH ON SULPHUR

Autotrophic growth on sulphur by LM7 showed an optimum temperature for growth of 45°C (Fig.31 and 32) with a doubling time of about 10 hours as estimated by sulphate production. At 50°C there was a rapid decline in growth rate. The influence of

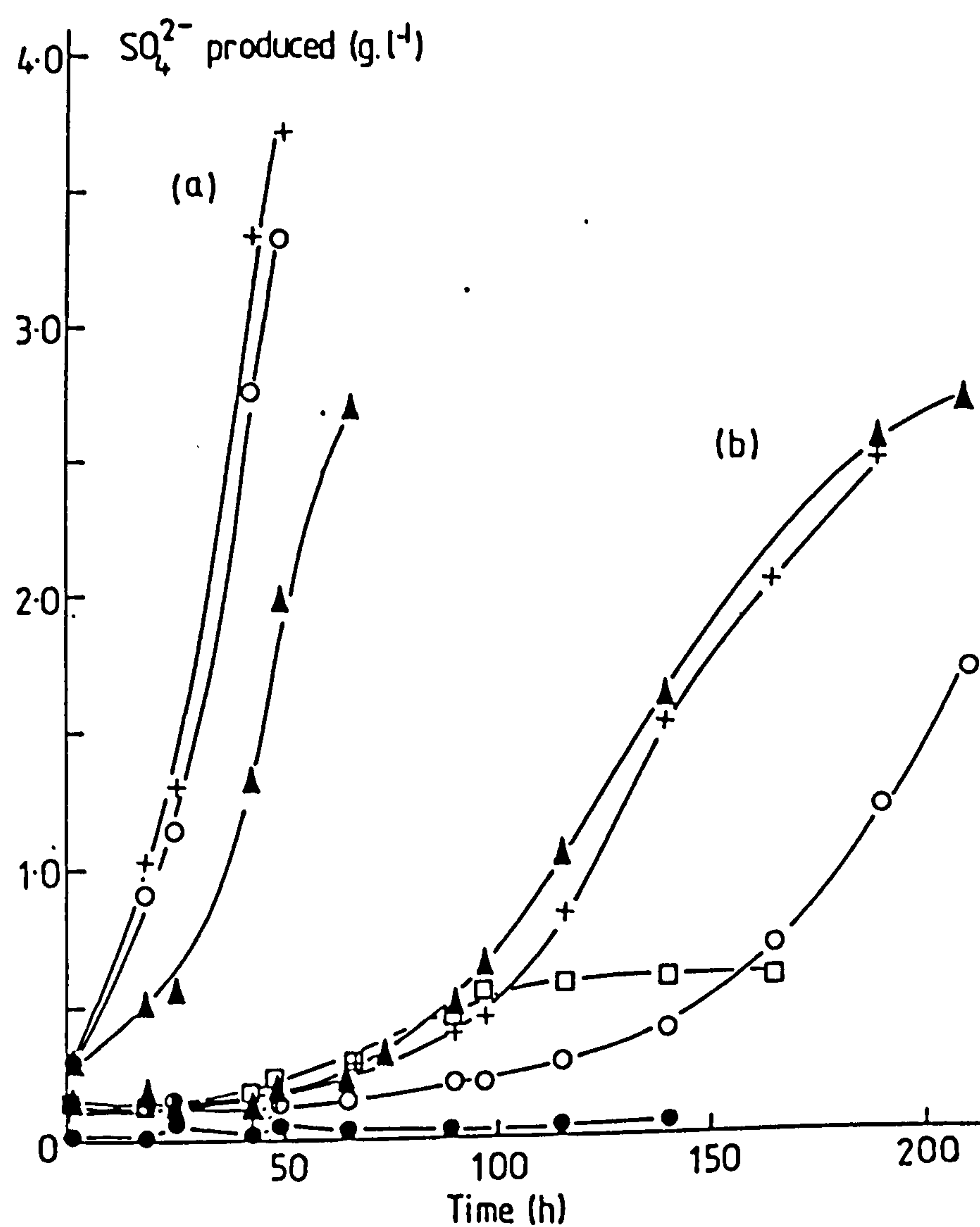


Fig. 31 Sulphate production during autotrophic growth of (a) LM7 and (b) LM2 on flowers of sulphur at 37°C (○), 45°C (+), 50°C (▲) and 55°C (□) with 5% (v/v) CO_2 in air flask atmospheres.

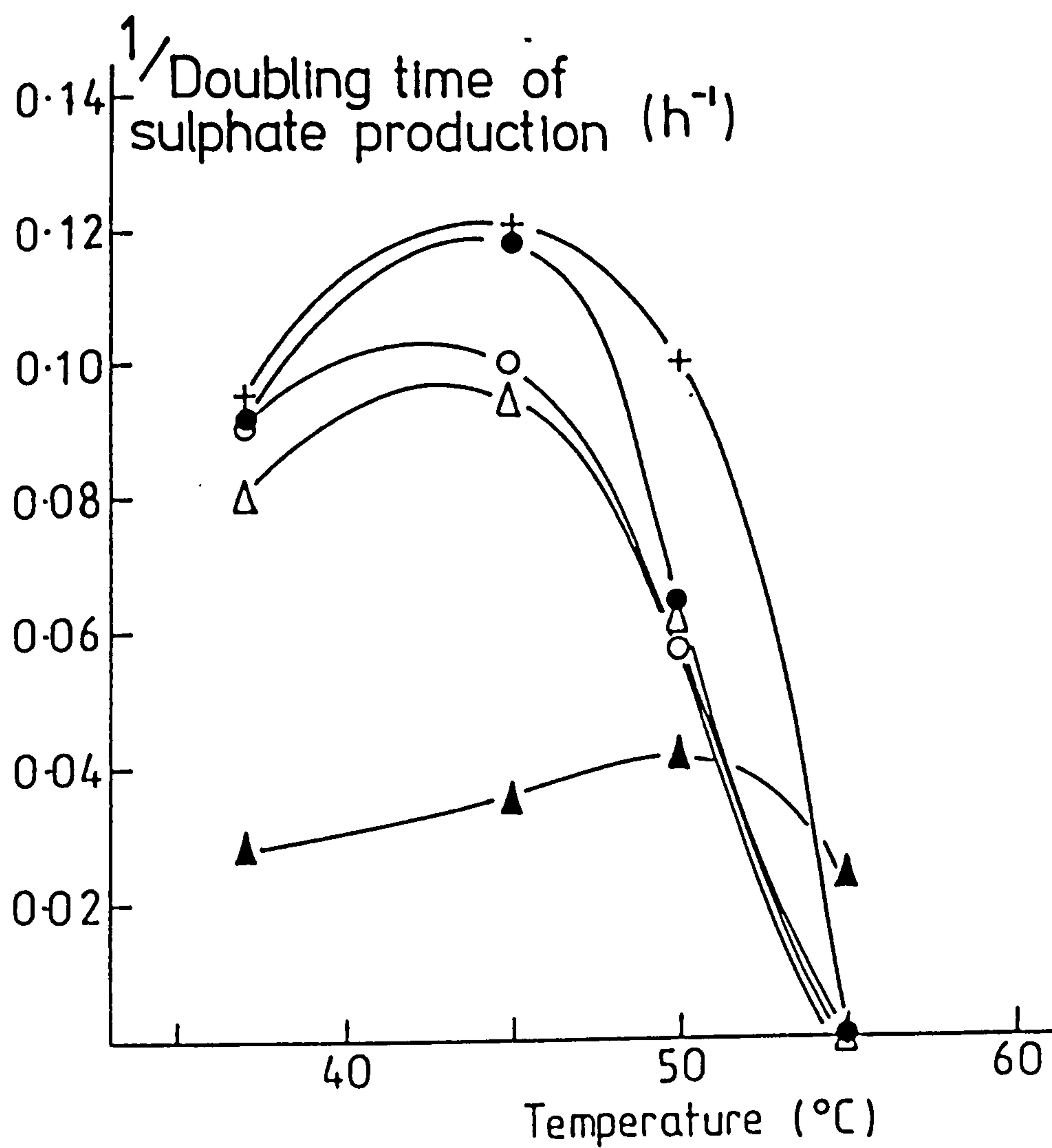


Fig. 32 The effect of temperature on growth of BC2 (●), BC4 (+), BC13 (Δ), LM7 (○) and LM2 (▲) on flowers of sulphur with 5% (v/v) CO₂ in air flask atmospheres.

temperature on growth followed a similar pattern with BC2, BC4 and BC13, all of which had an optimum temperature for growth of 45°C and maximum doubling times, when measured by sulphate production, from 8.3 to 10.5 hours (Fig.32).

In contrast, LM2 showed an optimum temperature for growth on sulphur of 50°C and a much slower doubling time of 25 hours. At 37°C there was a long lag phase before the faster rate of growth was obtained and at 55°C growth was inhibited.

All rates were obtained from shake flask data and slightly faster rates have been observed when these organisms were grown in 300 ml stirred culture vessels.

Isolates BC2, BC4, BC13 and LM7 were unable to oxidize ferrous iron at any temperature but LM2 readily showed the capacity to oxidize iron.

The newly-isolated, moderately thermophilic iron-oxidizing bacteria from Birch Coppice, Evenwood, Kingsbury and Lake Myvam (see Section 3.4) did not oxidize elemental sulphur on transfer to the sulphur salts medium from iron-oxidizing cultures.

5.5 MORPHOLOGY

The sulphur oxidizers BC2, BC4, BC13 and LM7 were all short, motile, rod-shaped bacteria resembling T. thiooxidans. Electron micrographs of LM7 (Fig.33a) showed them to measure 1.3 to 2.0 μm by 0.7 μm . The presence of a single polar flagellum was observed in isolate BC13 (Fig.34) at a 25,000 times magnification. The cell was heavily stained with uranyl acetate to reveal the presence of the flagellum.

In contrast, the sulphur-oxidizer LM2 was a non-motile,

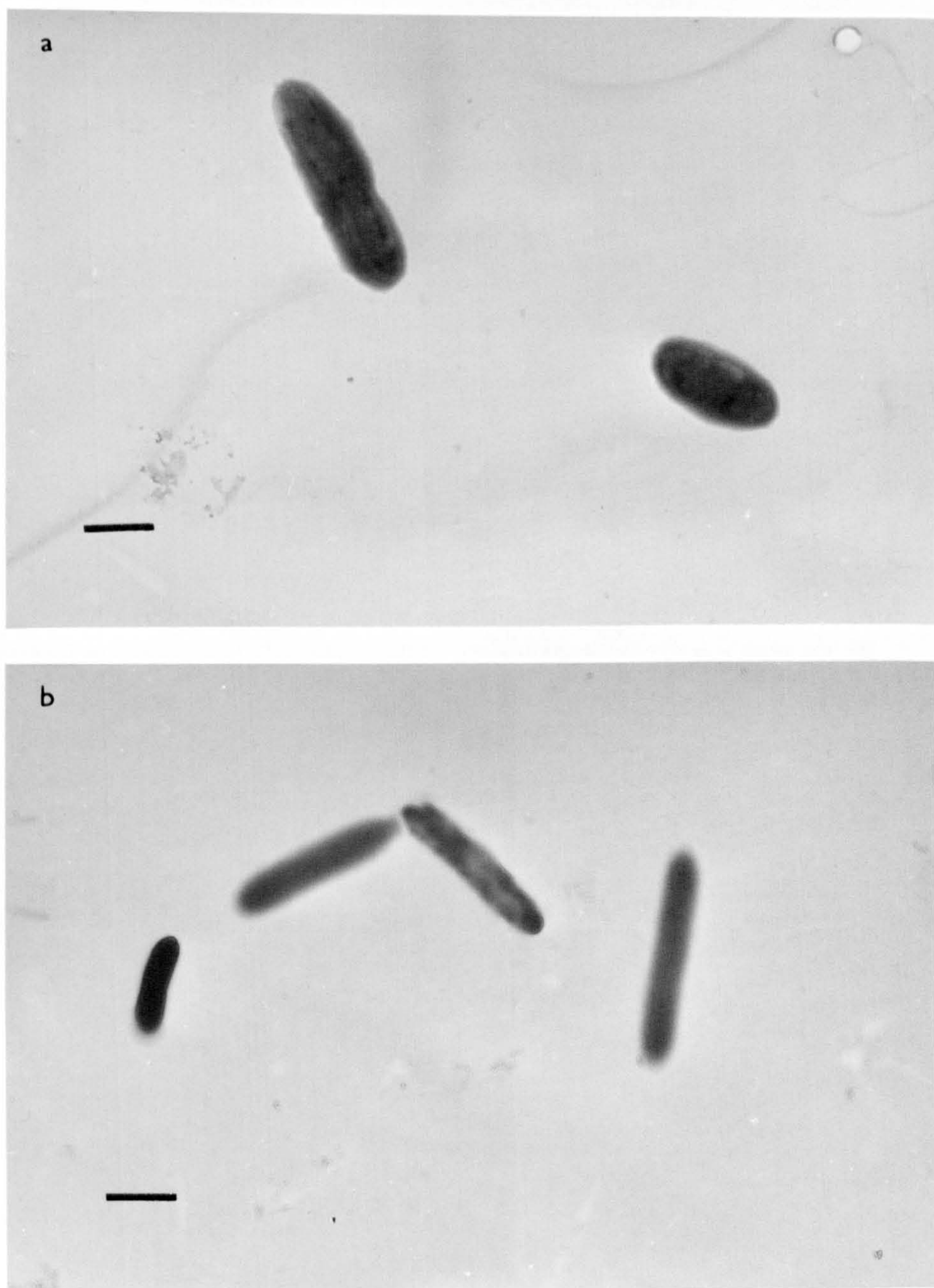


Fig. 33 Moderately thermophilic bacteria LM7 (a) and LM2 (b) grown autotrophically on flowers of sulphur. The cells were fixed but not stained. Bar marker, 1 μm .

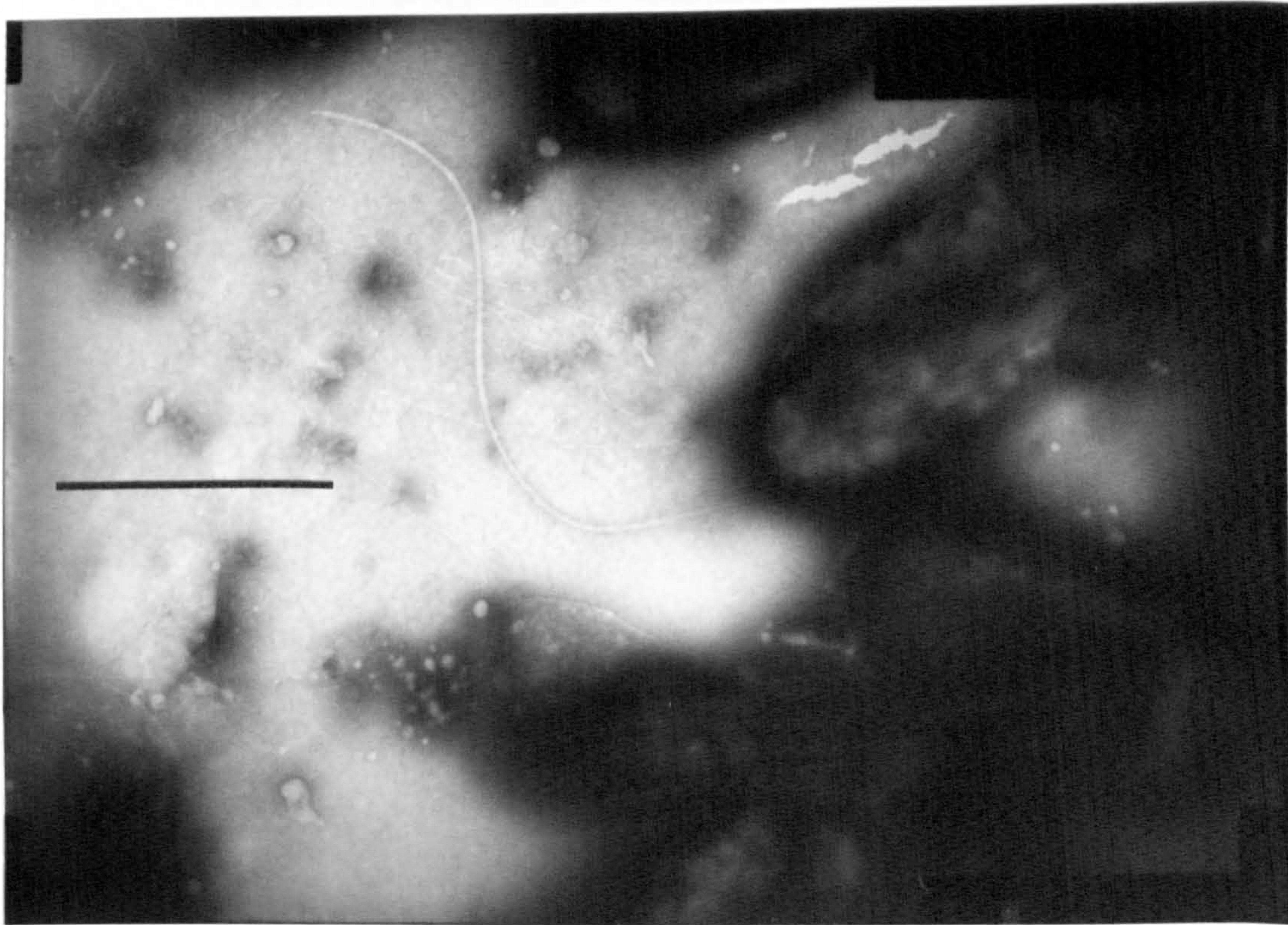


Fig. 34 The moderately thermophilic sulphur-oxidizing BCl3 stained with uranyl acetate to show a single polar flagellum. Bar marker, 1 μ m.

rod-shaped bacterium resembling the TH1 iron-oxidizing type and measuring 1.5–3.0 μm by 0.5 μm (Fig.33b).

5.6 COMPARISON OF THE ISOLATES BY ELECTROPHORESIS OF CELL PROTEINS

For comparative purposes, eight of the major protein bands of isolate BC13 (—), i.e. the dark bands in the central portion of the gel (Fig.35), were selected as marker bands. Six of the eight marker bands of T. thiooxidans (—+) were in common with those from BC13. Isolate BC4 had the same eight major protein bands as BC13 with positional changes in some of the minor bands. Isolate BC2 showed more variation with only four bands in common with BC13 and the three major protein bands in different positions (+). LM7 only showed three common protein bands with BC13, other major bands being in different positions (<). LM2, although a fainter preparation, had three bands in common with BC13 (—) but none in common with T. thiooxidans.

A comparison of the electrophoregrams when LM2 and the Lake Myvam iron-oxidizing bacterium (LM1) were grown autotrophically on ferrous iron (Fig.36) showed that out of 10 bands from LM2, 5 were common to LM1 (—) and 5 were absent (—+).

5.7 DISCUSSION

The dominant, moderately thermophilic sulphur-oxidizing bacteria (i.e. those that were most readily isolated and oxidized sulphur most rapidly) obtained from the coal-rich and hot spring samples could not oxidize ferrous iron. They therefore, physiologically and morphologically, resemble the "high

Fig. 35 Electrophoretic protein patterns of whole cell lysates of sulphur-oxidizing bacteria, T. thiooxidans (A & G), LM7 (B), LM2 (C), BC13 (D), BC4 (E) and BC2 (F).

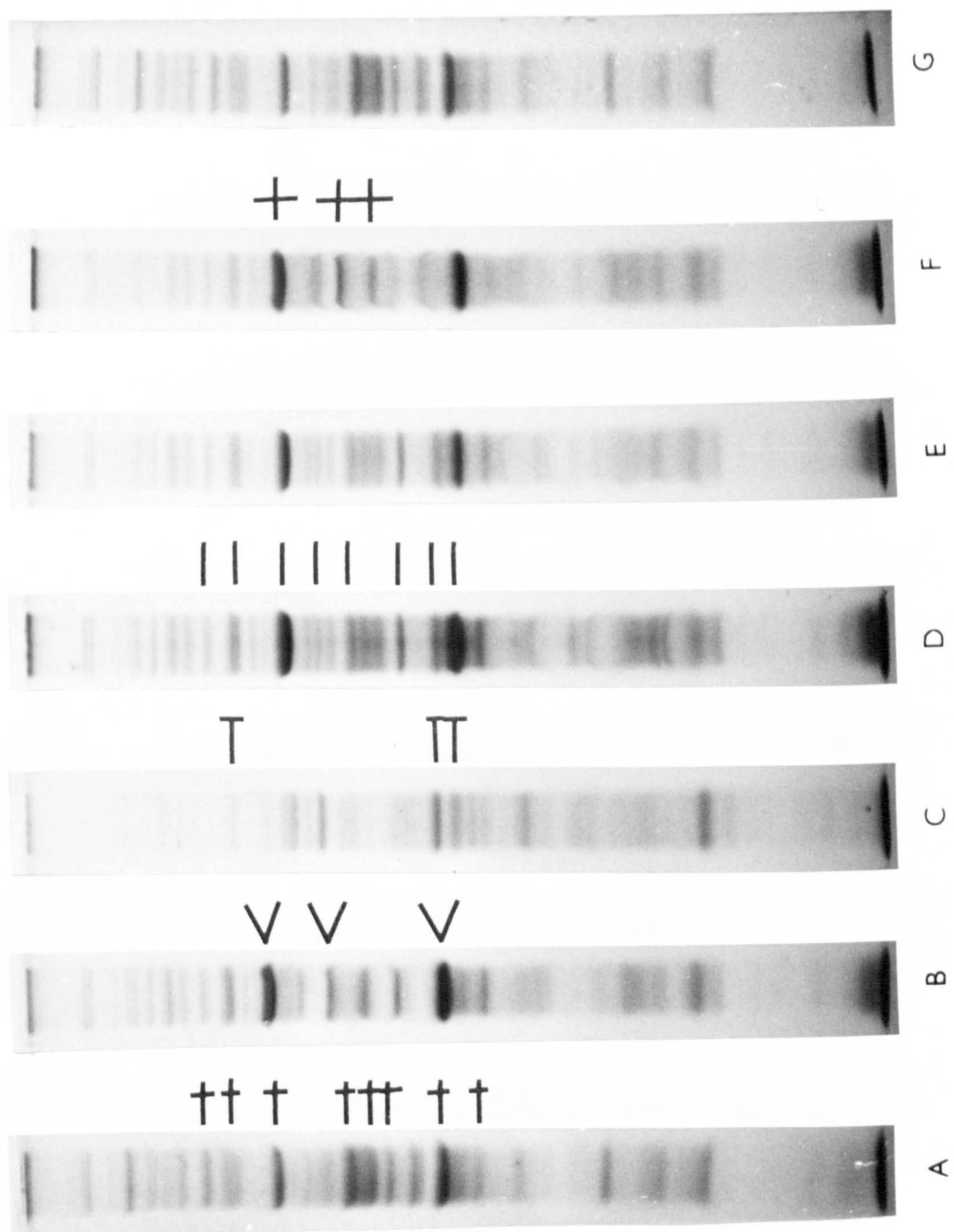
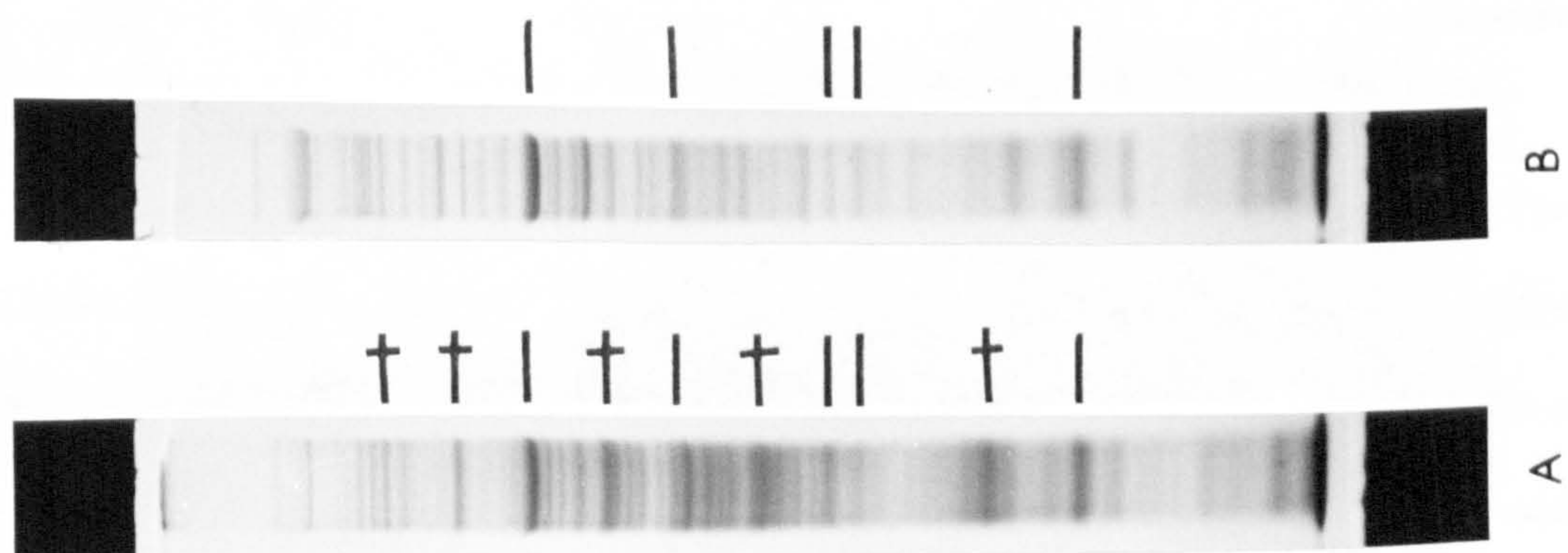


Fig. 36 Electrophoretic protein patterns of whole cell lysates of LM2 (A) and LM1 (B) grown autotrophically on ferrous iron.



temperature" strains of T.thiooxidans which have been observed previously (Schwartz & Schwartz, 1965; Schoen & Ehrlich, 1968; Fliermans & Brock, 1972).

The electrophoresis patterns confirmed a similarity between T.thiooxidans and BC13, BC2, BC4 and LM7. However there were more variations observed among the electrophoresis patterns of this 'group' of sulphur-oxidizers than was evident among the moderately thermophilic iron-oxidizing bacteria (see Section 3.7) indicating a greater variation among the sulphur oxidizers. The electrophoregrams were also useful in showing that similar sulphur-oxidizing bacteria can be isolated from a site despite the different enrichment temperatures, e.g BC2 at 50°C and BC13 at 37°C. However, the electrophoregrams do not adequately show the degree of similarity between the new isolates. A DNA-DNA homology study similar to that performed with strains of T. thiooxidans (Harrison, 1982) would enable the taxonomic position of the isolates to be resolved.

The largest deviation from the T. thiooxidans type electrophoresis pattern occurred with the LM2 isolate. This isolate also differed in morphology, growth substrate utilization in solid medium, optimum temperature for growth of 50°C and the doubling time for growth on sulphur which was approximately 3 times slower than that of the other isolates. LM2 was the only moderately thermophilic organism isolated to consistently show both sulphur and iron oxidation at 50°C and is clearly different from both the solely sulphur-oxidizing LM7 and the solely iron-oxidizing bacterium LM1 (see Section 3.4) from the Lake

Myvam site. More detailed physiological experiments are required to characterize this organism, particularly with regard to its iron-oxidizing capacity. The LM2 isolate also requires comparative DNA-DNA homology studies with both the moderately thermophilic sulphur-oxidizing bacteria and the iron-oxidizing bacteria to determine its relationship with these two 'groups' of moderately thermophilic bacteria.

All the isolates in this study were grown on sulphur under autotrophic conditions and so further investigation is required to determine whether they are capable of mixotrophic growth on sulphur or heterotrophic growth on organic compounds.

The growth of TH1 on sulphur has been described (Fig.30a) yet the failure of the other TH1-like bacteria to grow on sulphur was also noted. Further work is clearly required on the sulphur-oxidizing potential of these moderately thermophilic iron-oxidizing bacteria. Firstly, the purity of the TH1 culture needs to be examined to ensure that no sulphur-oxidizing bacteria of the LM7 or BC4 types are present. Secondly a wide variety of conditions (e.g. variations in inoculum size and agitation rate) should be used in further attempts at establishing transfer of iron-oxidizing cultures to sulphur-containing media. Growth of the Birch Coppice TH1-like organism on chalcopyrite (see Section 7.3) could indicate that the organism does have the capacity for sulphide or sulphur oxidation as other bacteria capable only of iron oxidation (e.g. Leptospirillum ferrooxidans) only degrade chalcopyrite in the presence of sulphur - oxidizing bacteria (Norris, 1983). Similarly, a final culture pH after chalcopyrite

leaching by the moderately thermophilic iron- oxidizing bacteria which was similar to, or lower than, the pH after chalcopyrite oxidation by T. ferrooxidans (see Section 7.3) could suggest that the thermophiles were responsible for at least as much sulphur oxidation as the iron and sulphur-oxidizing mesophile during the mineral dissolution.

Another possible explanation for the lack of sulphur oxidation by the TH1-like bacteria could lie in the stock culture maintenance procedures. The growth of TH1 on sulphur was achieved at the start of this work when the organism had been recently maintained on a nickel concentrate. The attempts to transfer the newly isolated TH1-like bacteria to a sulphur-containing medium followed their maintenance on ferrous iron or pyrite for almost two years. It is not known whether their maintenance in the absence of sulphur, on iron or pyrite (which can be microbially degraded in the absence of bacterial sulphur oxidation (Norris, 1983)) could result in the loss of a sulphur- oxidizing capacity.

P A R T 6

EXTREMELY THERMOPHILIC IRON- AND SULPHUR-OXIDIZING ACIDOPHILES

6.1 INTRODUCTION

Strains of Sulfolobus were originally described after their isolation from Yellowstone National Park, U.S.A. by Brierley (1966). Considerable study of their habitat, ecology and growth (Brock et al., 1972; Brock, 1978) showed them to be facultatively autotrophic with growth occurring on sulphur and on a variety of simple organic compounds. The pH optimum of these organisms was 2.0-3.0 although they occurred over a range from 0.9-5.8. Over the temperature range 55°-84°C three strains were characterised with the optimum temperature of each strain being dependent on both the habitat and isolation temperatures (Mosser et al., 1974). Immunochemical studies on the cell surfaces of these strains confirmed the groupings based on physiological data (Bohloul & Brock, 1974). Such organisms are thought to contribute to the high level of sulphuric acid production in natural, acidic, geothermal habitats (Fliermans & Brock, 1972; Mosser, 1973).

From these studies the type strain S. acidocaldarius was described which had a temperature optimum of 70-75°C and a pH optimum of 2.0-3.0 (Brock et al., 1972). The doubling time for growth on sulphur varied between 37-55 hours dependent on strain (Shivvers & Brock, 1973). Growth occurred more rapidly on sulphur in the presence of yeast extract although sulphur oxidation was partially inhibited (Shivvers & Brock, 1973). S. acidocaldarius 98-3 also grew on a variety of organic substrates but not on glucose (Brock et al., 1972). The doubling time for growth on yeast extract (1.0 g.l^{-1}) was reported as 6.5

- 8.0 hours (Brock et al., 1972).

Other Sulfolobus-type organisms have since been isolated from hot springs and thermal acid soils around the world (Bohloul, 1975; Furuya et al., 1977) including the M.T. strains and S. solfataricus from Agnano, Italy (DeRosa et al., 1975; Zillig et al., 1980), S. brierleyi (Brierley & Brierley, 1973) (previously ferrolobus (Langworthy, 1977)) and Sulfolobus B6-2 (Konig et al., 1982). S. solfataricus and S. brierleyi have been described as different species to S. acidocaldarius on the basis of their RNA polymerase sub-unit composition (Zillig et al., 1980). S. solfataricus has been grown on sulphur, yeast extract and a variety of sugars between 50-87°C with a temperature optimum of 87°C and a pH range of 3.5-5.0 (Zillig et al., 1980). Growth of S. brierleyi with ferrous iron as an energy source has been demonstrated; the rate of iron oxidation was enhanced in the presence of yeast extract (Brierley & Brierley, 1973).

In natural populations, where the total iron concentration ranged from 1-200 ppm, S. acidocaldarius was shown to oxidize ferrous iron. However the ratio of ferric to ferrous iron in a given spring pool depended on the rate of ground water flow into the pool, the rate of conversion from Fe^{3+} to Fe^{2+} as well as on the bacterial oxidation rate (Brock et al., 1976). S. acidocaldarius was also able to reduce Fe^{3+} back to the Fe^{2+} state using the sulphur present in the sediment as an electron donor (Brock & Gustafson, 1976).

Although spherical in nature some isolates have been reported to be more lobate than others (DeRosa et al., 1974).

Cell shape was also demonstrated to be growth phase-dependent (McClure & Wyckoff, 1982) but both these observations might have been due to artefacts occurring in preparation rather than real morphological variations (Millonig et al., 1975). The isolates have been reported as having diameters of 0.7-1.5 μm for the M.T. strains (Millonig et al., 1975), 1.0-1.5 μm for S. brierleyi (Brierley & Brierley, 1973) and 0.8-2.0 μm for S. acidocaldarius 98-3 and S. solfataricus (Zillig et al., 1980). S. acidocaldarius has been shown to produce adhesive pili which enabled it to attach to sulphur crystals (Weiss, 1973). Weiss has shown that although attachment is not a prerequisite for sulphur oxidation the presence of pili does enable colonization in hot, acid streams as they are heat stable upto 75°C and pH stable to 2.0 (Weiss, 1973). S. acidocaldarius is also capable of adhesion using the cell wall only (Weiss, 1973).

The cell surface of Sulfolobus strains has been shown to be devoid of peptidoglycan (Weiss, 1974; Langworthy, 1977) and to possess characteristic lipids (Langworthy, 1977) which could be adaptations to living in the extreme environment of low pH and high temperature.

Various guanine plus cytosine (GC) contents of DNA have been reported in the literature for members of the Sulfolobus group (Brock et al., 1972; Brierley & Brierley, 1973; DeRosa et al., 1975) but one comparative study (Zillig et al., 1980) showed that S. acidocaldarius 98-3 had a content of 41 mol% GC, S. solfataricus DSM 1616 a content of 40 mol % and S. brierleyi a content of 37 mol %.

Thermoplasma acidophila (Darland et al., 1970; Brock, 1978) has been isolated from coal refuse piles and is also a spherical, extremely thermophilic organism. However it is less thermophilic than Sulfolobus having an optimum temperature of 59°C and is incapable of autotrophic growth. It has no cell wall and a lower GC content of 24-29 mol % (Darland et al., 1970; DeRosa et al., 1974; Brock et al., 1972).

A comparative study between existing and newly isolated Sulfolobus-type organisms was undertaken to establish some of the growth substrates that each organism was capable of utilizing. As with the physiological growth studies on the moderately thermophilic bacteria (see Sections 3 and 5) the emphasis was placed on the chemolithoautotrophic growth on iron and sulphur rather than heterotrophic or chemolithoheterotrophic growth. Together with electrophoregrams of the isolates proteins, the aim was to give a clearer understanding of the relationships between the different Sulfolobus-type organisms.

6.2 ISOLATION OF EXTREMELY THERMOPHILIC BACTERIA

When enrichment cultures from Lake Myvam and Birch Coppice were plated onto thiosulphate and tetrathionate agar plates (see Section 2.7.3) in the absence of organic nutrients and incubated at 65°C small colonies were visible after approximately 2 weeks. However, the transfer of single colonies into a liquid medium did not result in further growth. The cultures from Lake Myvam and Birch Coppice were therefore maintained as enrichment cultures.

6.3 GROWTH ON YEAST EXTRACT

Rapid growth on yeast extract occurred with S. acidocaldarius and S. solfataricus (Fig.37 & 38). S. acidocaldarius 98-3 showed an increase in the rate of growth as the temperature was increased from 60°-77°C (Fig.39) and so the optimum temperature was not reached in these experiments. A maximum doubling time for growth (measured as optical density) on yeast extract (0.5 g.l⁻¹) of 4 hours was reached at 77°C by S. acidocaldarius 98-3. S. solfataricus increased its rate of growth on yeast extract from 60°-69°C but the rate decreased beyond 73°C (Fig.39). A maximum doubling time for growth at 69°C on yeast extract was approximately 6 hours. The enrichment culture Sulfolobus (Lake Myvam) grew slowly on yeast extract in the first sub-culture from an iron-grown culture at 70°C but more rapidly in the second sub-culture with a doubling time of approximately 4.0 hours. When an inoculum from the third sub-culture on yeast extract was transferred back into ferrous iron medium no growth or iron oxidation occurred.

6.4 GROWTH ON FERROUS IRON

S. acidocaldarius 98-3, S. solfataricus and Sulfolobus B6-2 did not oxidize iron at 70°C under autotrophic conditions or in the presence of yeast extract in shake flask experiments. Serial sub-cultures of S. brierleyi on ferrous iron supplemented with yeast extract grew extensively with reproducible Fe²⁺ oxidation over many sub-cultures (Fig.40). The doubling time for iron oxidation was approximately 10 hours at 70°C when measured over the 10-20% oxidation phase. When S. brierleyi was grown without

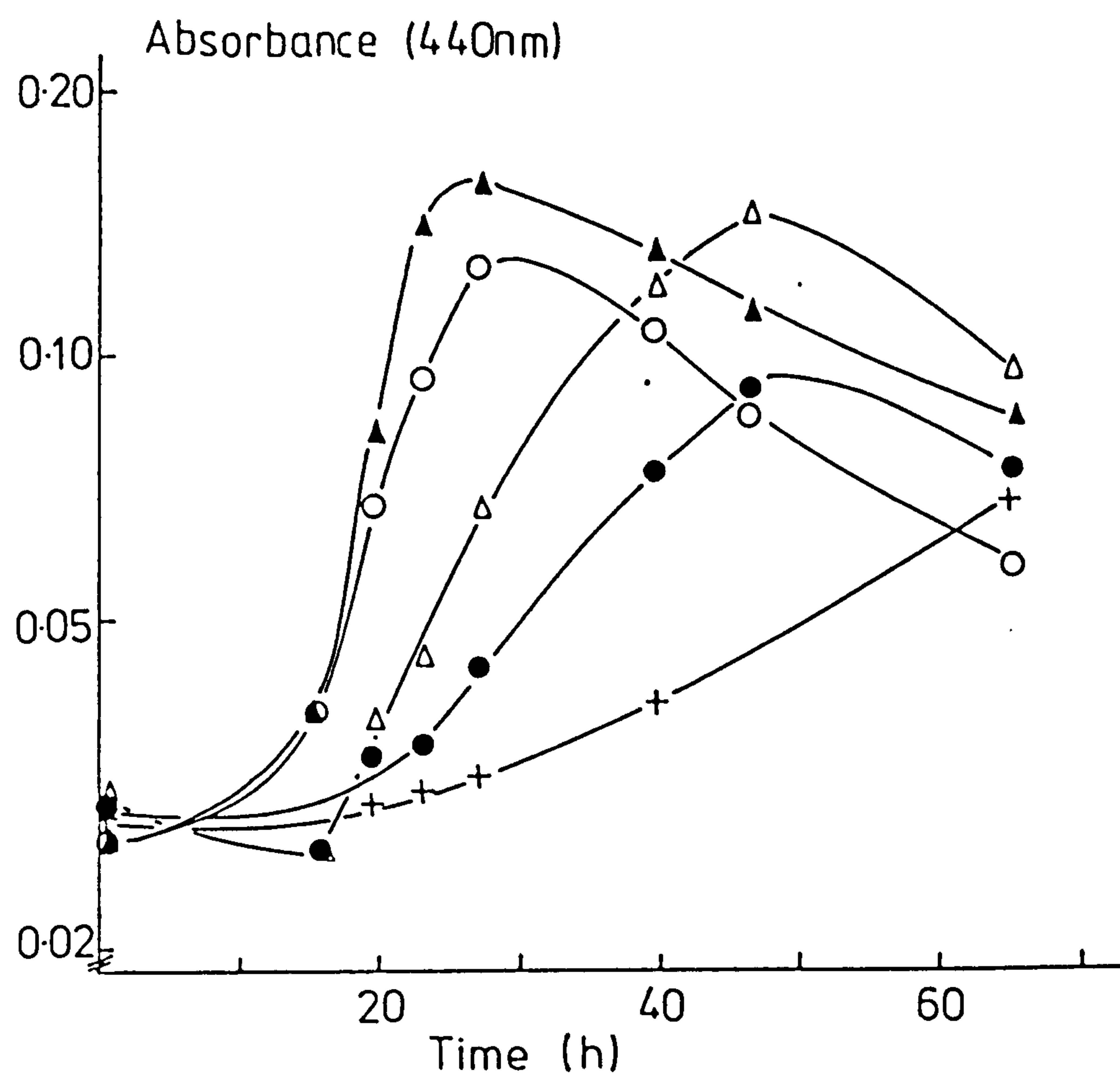


Fig. 37 The growth of *S. acidocaldarius* 98-3 at 60°C (+), 65°C (●), 69°C (Δ), 73°C (○) and 77°C (▲) on yeast extract (0.5g.l⁻¹).

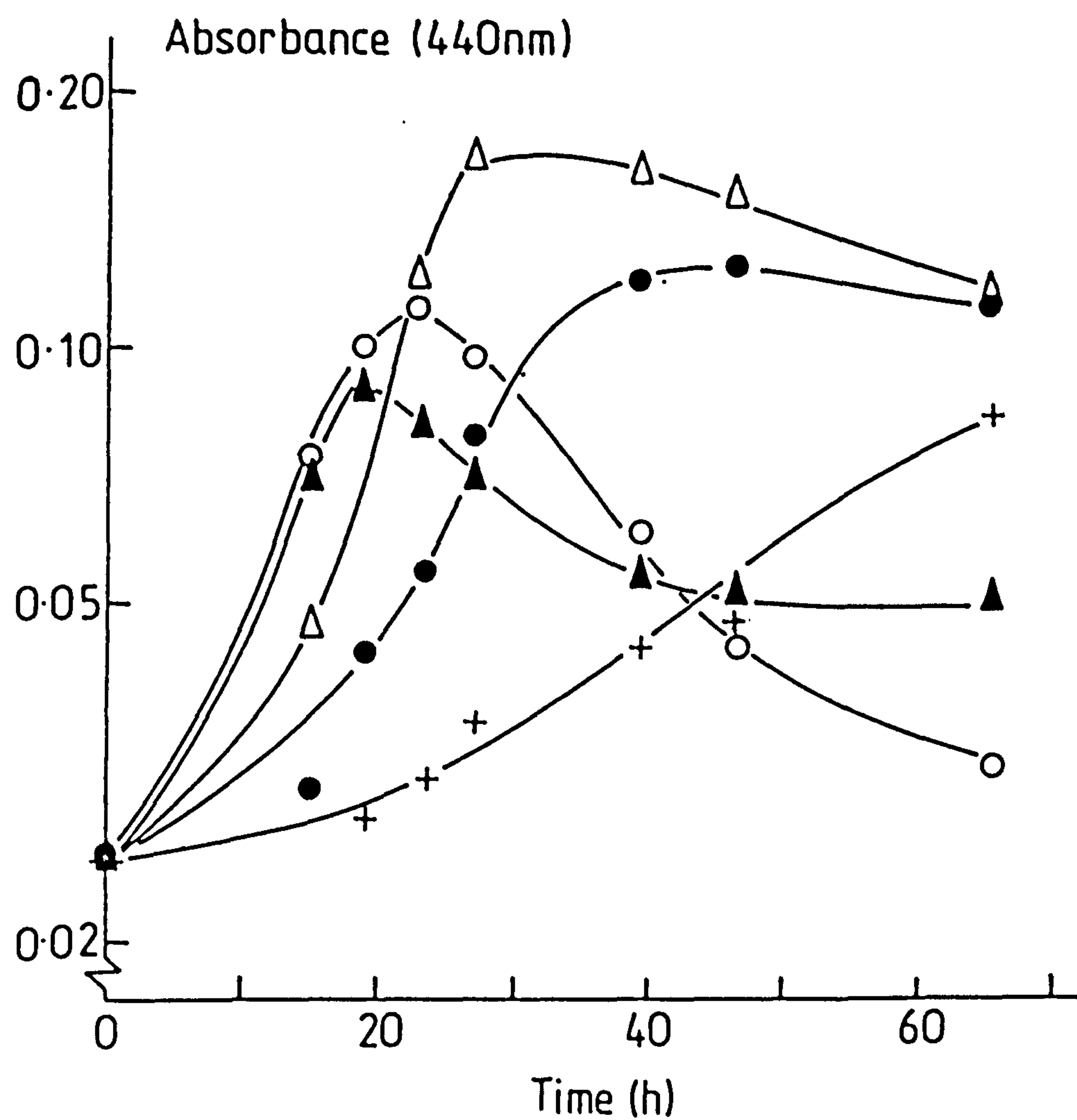


Fig. 38 The growth of *S. solfataricus* at 60°C (+), 65°C (●), 69°C (Δ), 73°C (○) and 77°C (▲) on yeast extract (0.5g.l⁻¹).

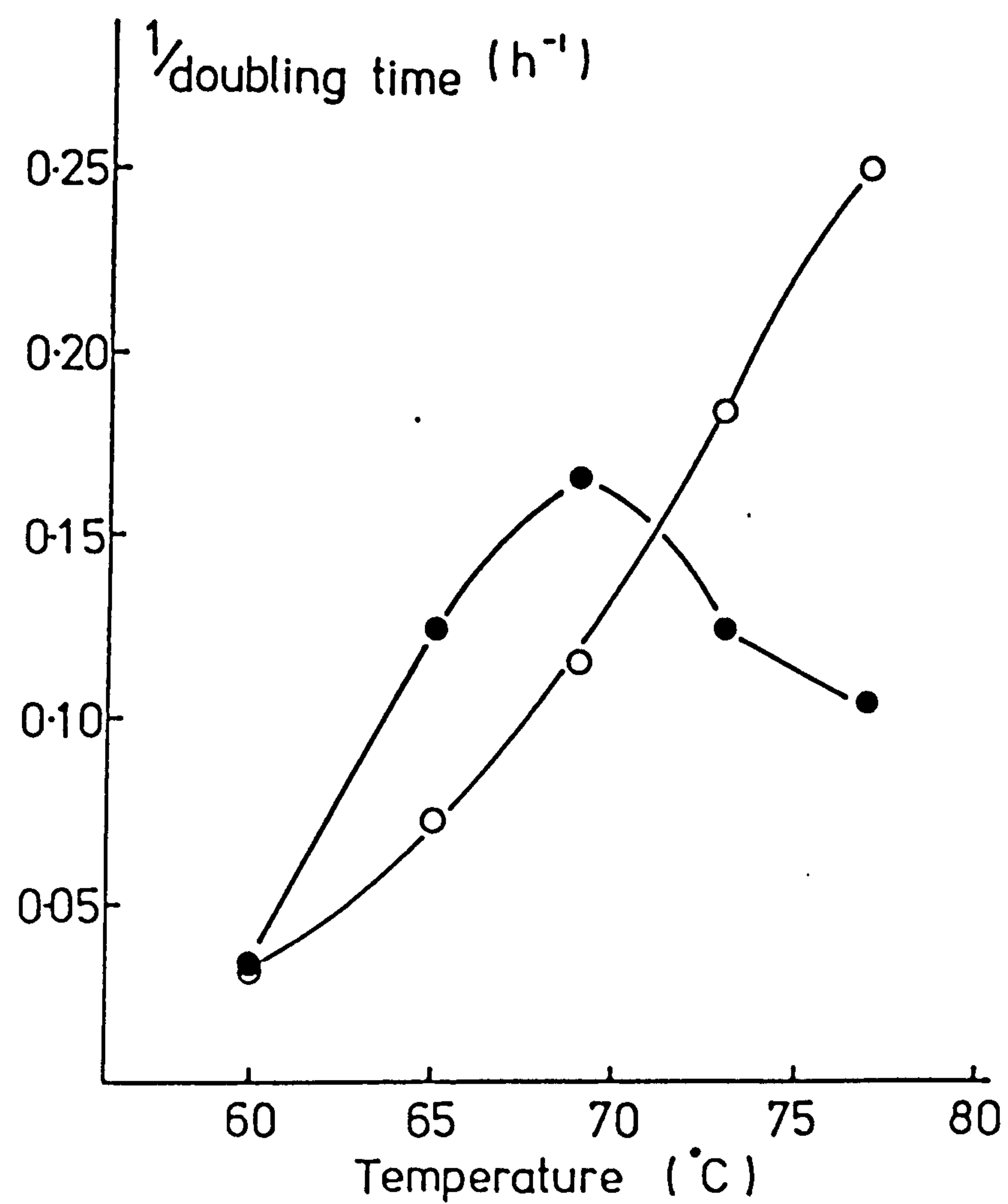
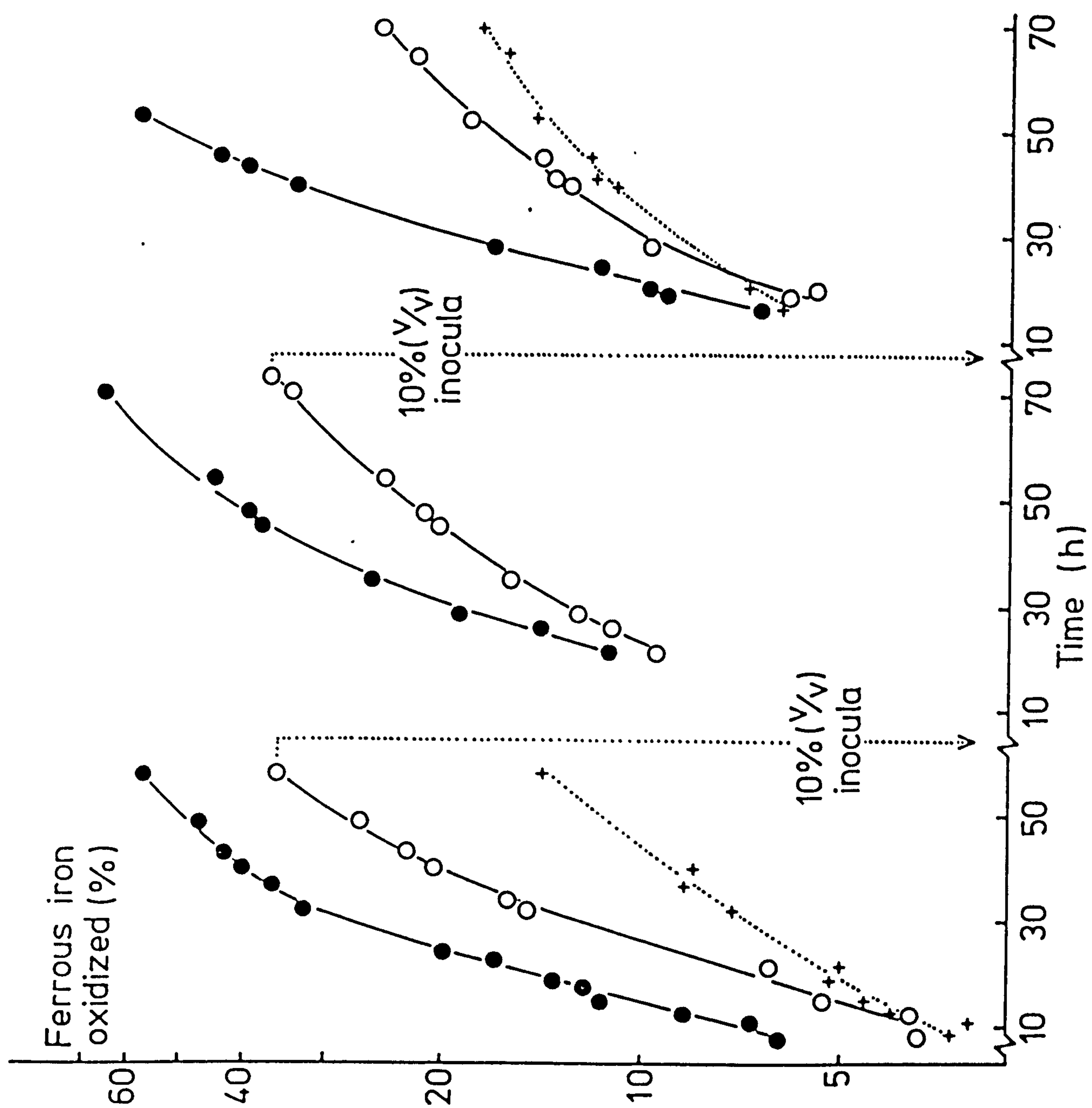


Fig. 39 The effect of temperature on the growth rates of *S. acidocaldarius* 98-3 (\bigcirc) and *S. solfataricus* (\bullet) on yeast extract.

Fig. 40 Iron oxidation at 68°C in sterile controls (+) and by Sulfolobus brierleyi in medium containing ferrous iron (50 mM) and thiosulphate through serial cultures with (●) and without (○) yeast extract. The initial inocula were from a yeast extract - supplemented culture and the subsequent inocula were taken, as indicated, from the cultures without yeast extract.



the yeast extract supplement and serially sub-cultured the rate of iron oxidation decreased with each sub-culture (Fig.40).

Sulfolobus (Lake Myvam) showed a slightly higher rate of iron oxidation in the presence of yeast extract than S.brierleyi with a doubling time for iron oxidation of approximately 8.0 hours at 70°C over the 10-20% oxidation phase with the rate being reproducible over many sub-cultures (Fig.41). Sulfolobus (Lake Myvam) also showed reproducible autotrophic iron oxidation over many sub-cultures. The inoculum used in the experiment described in Fig.41 came from the tenth serial sub-culture on ferrous iron in the absence of yeast extract. The doubling time for iron oxidation was 9.0 hours. The omission of thiosulphate from autotrophically growing cultures resulted in a shortening of the exponential phase of growth even though traces of thiosulphate would have been present due to carryover in the inoculum. Growth on ferrous iron at 70°C was observed with Sulfolobus (Birch Coppice) and S. acidocaldarius (from J. Brierley) but no detailed growth determinations were performed.

6.5 GROWTH ON SULPHUR

S. acidocaldarius 98-3 and S. solfataricus showed a visible optical density increase and high cell concentrations under microscopic examination but an increase in culture pH when grown on sulphur in the presence of yeast extract (Fig.42). Under identical conditions Sulfolobus (Lake Myvam) and Sulfolobus (Birch Coppice) showed substantial growth accompanied by a rapid fall in culture pH (Fig.42). A similar fall in pH was observed by these latter cultures during autotrophic growth on sulphur.

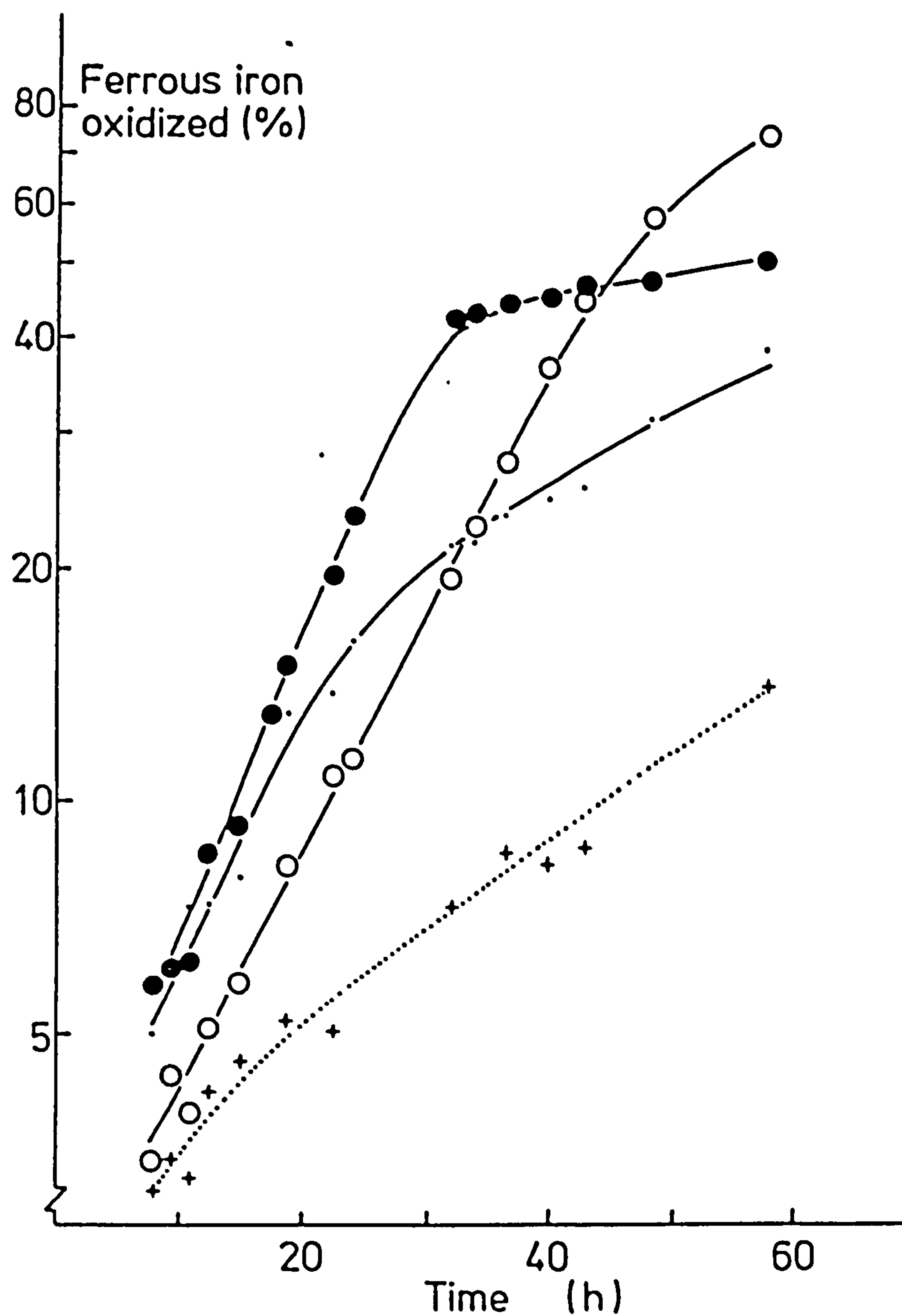


Fig. 41 Iron oxidation at 68°C in a sterile control (+) and by Sulfolobus (Lake Myvam) in medium containing ferrous iron (•) and ferrous iron plus thiosulphate (○) or yeast extract (●). Inocula (10% v/v) were from a ferrous iron and thiosulphate-supplemented culture.

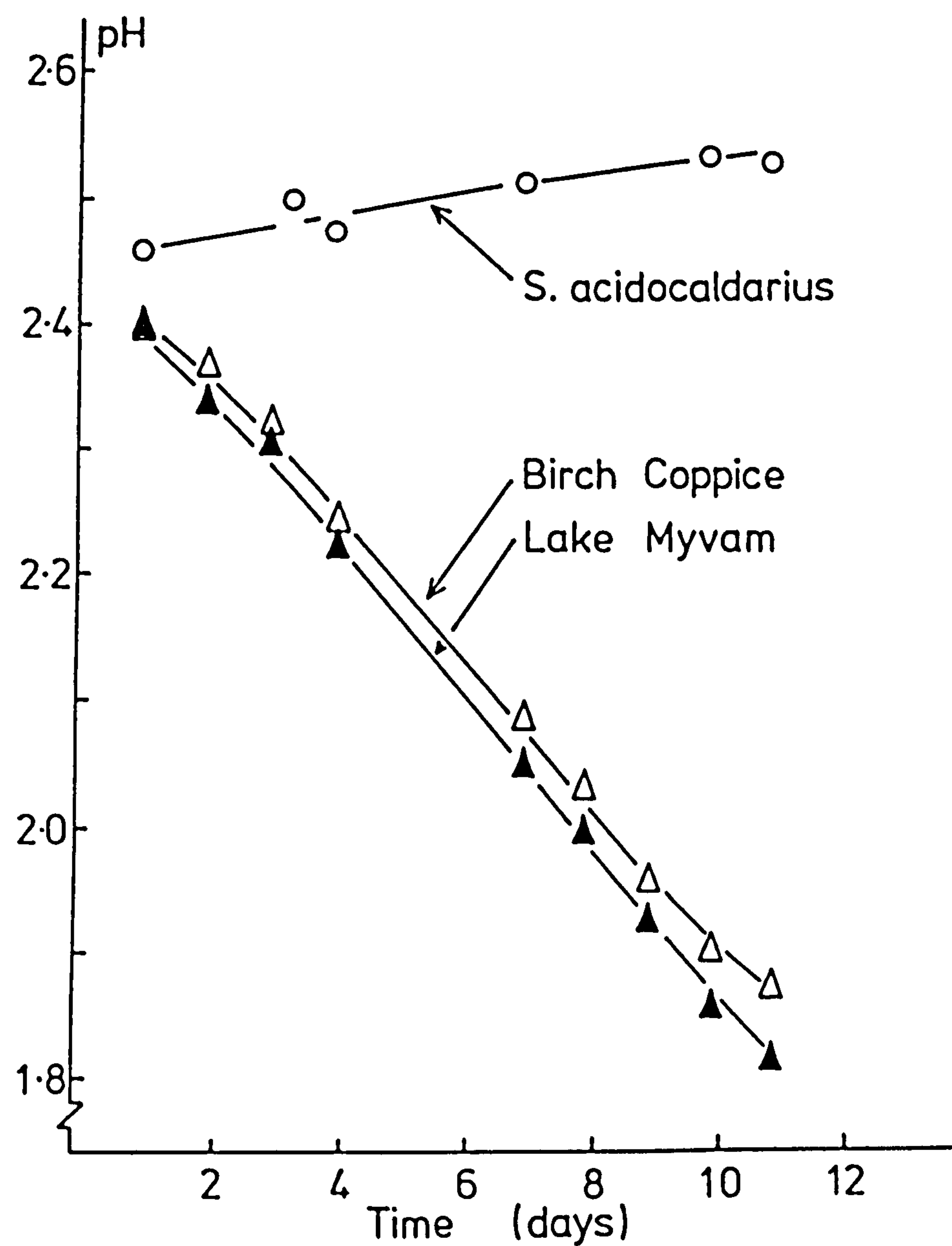


Fig. 42 The pH of the sulphur salts medium plus sulphur and yeast extract during growth at 70°C of *S. acidocaldarius* 98-3 and *Sulfolobus* enrichment cultures from Birch Coppice and Lake Myvam.

The doubling time for sulphate production by Sulfolobus (Lake Myvam) when measured in stirred, aerated vessels at 68°C was under 10 hours (Fig.43). This rate is similar to that observed with the Thiobacillus-type sulphur oxidizing bacteria isolated from the same site (Fig.44).

The effect of temperature on iron, sulphur and pyrite oxidation by Sulfolobus (Lake Myvam)(Fig.43) shows optimum autotrophic growth occurring between 68°C and 71°C for all substrates. The ferrous iron oxidation curve has not been corrected with sterile controls so its value is as a guide to optimum temperature and not to specific rates.

Sulfolobus B6-2, S. brierleyi and S. acidocaldarius (from J. Brierley) also showed autotrophic growth on elemental sulphur but detailed determinations were not performed.

6.6 MORPHOLOGY

Cells of all isolates were spherical and occurred singly or in pairs. Variations between different cultures were not readily apparent under the light microscope. The size of the cells from one culture were uniform on a given substrate but varied between growth substrates; the smallest being observed during pyrite oxidation. Sulfolobus (Lake Myvam) when grown autotrophically on pyrite (see Section 7.4) had a cell diameter of 1.5-2.0 μm (Fig.45).

6.7 ELECTROPHORESIS OF CELL PROTEINS

S. acidocaldarius 98-3 and S. solfataricus could only be compared electrophoretically with the Lake Myvam culture grown on yeast extract and not with the other isolates or strains because

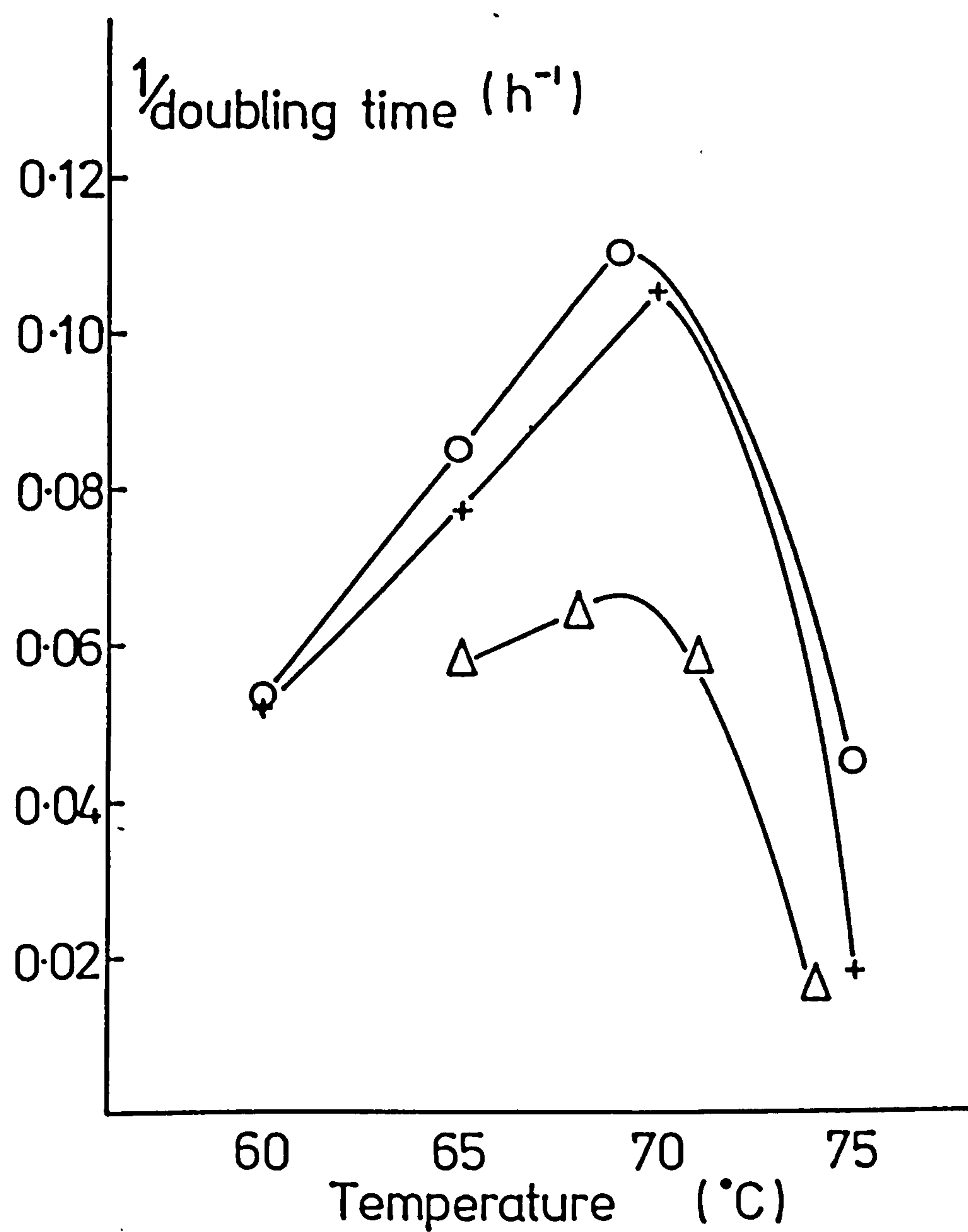


Fig. 43 The effect of temperature on autotrophic growth of Sulfolobus (Lake Myvam) during oxidation of iron (O), sulphur (+) and pyrite (Δ).

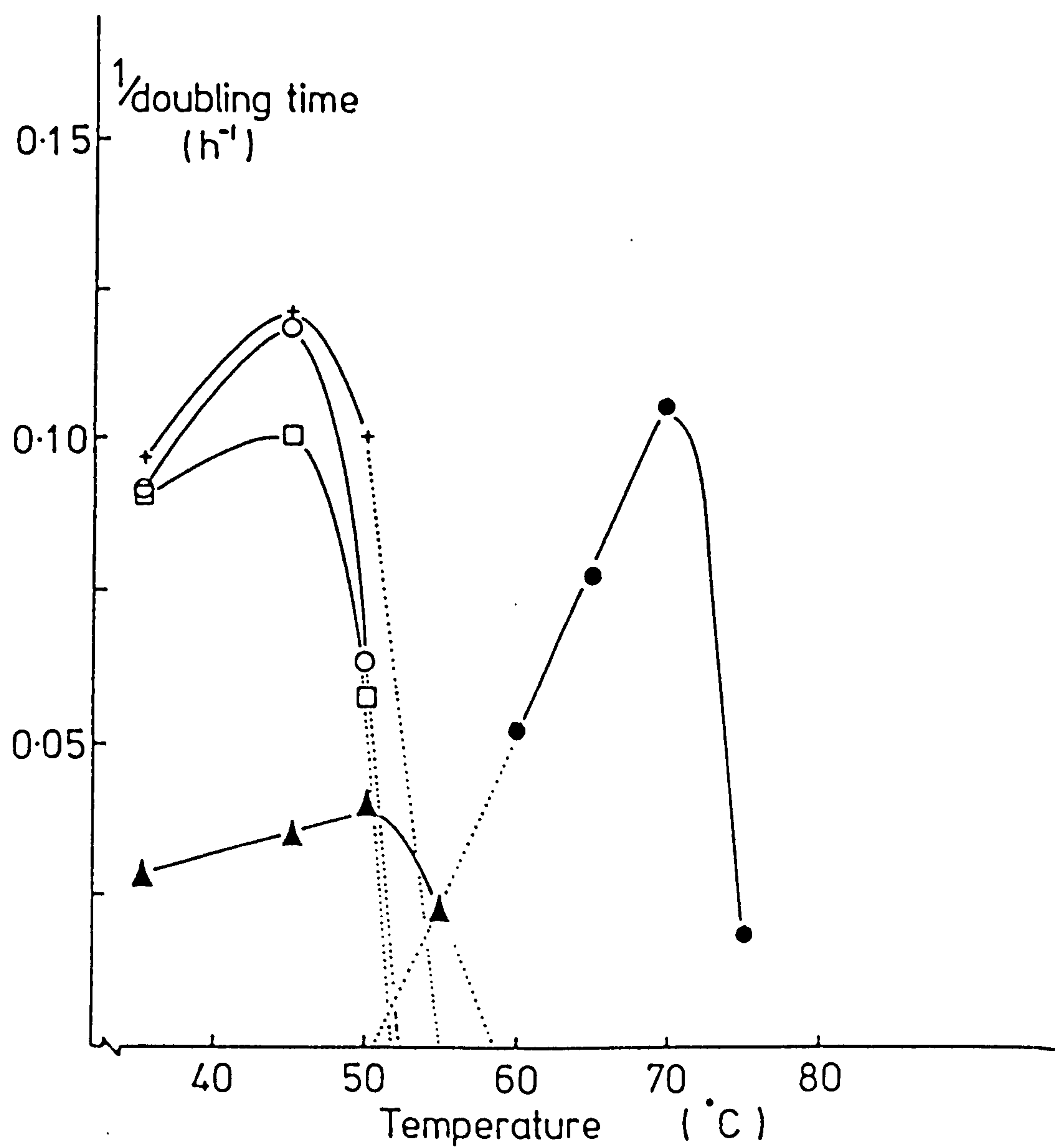


Fig. 44 The effect of temperature on the rate of sulphate production from sulphur during autotrophic growth of *Sulfolobus* (Lake Myvam, ●) and moderate thermophiles LM2 (▲), LM7 (□), BC4 (+) and BC2 (○).

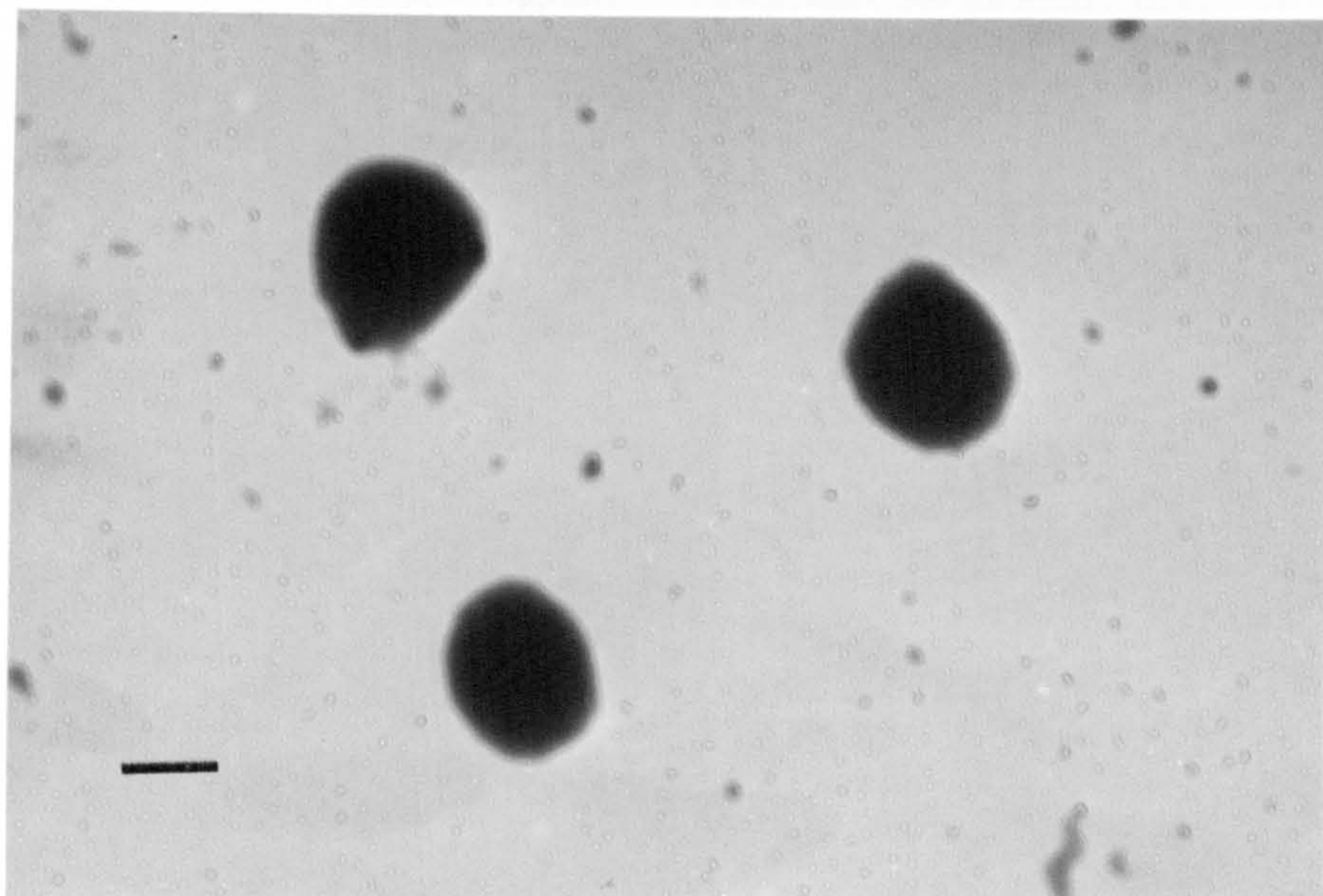


Fig. 45 Sulfolobus (Lake Myvam) grown autotrophically on pyrite.
The cells were fixed but not stained. The bar marker represents 1 μm .

of the need to make comparisons only between cells grown on the same substrates. Electrophoresis of whole cell proteins from the yeast extract-grown cultures of S. acidocaldarius, S. solfataricus and Sulfolobus (Lake Myvam)(Fig.46) showed that all 10 of the major protein bands of S. acidocaldarius (—) were also possessed by S. solfataricus and 8 were identified in Sulfolobus (Lake Myvam)(—+).

The Sulfolobus (Lake Myvam) culture capable of autotrophic growth was grown on pyrite, sulphur and tetrathionate. All 10 of the major protein bands (—) found in the tetrathionate-grown cultures were also present in the sulphur-grown cells and 8 were present in the pyrite-grown cells (—+)(Fig.47).

When cells from the Sulfolobus (Lake Myvam) culture grown on tetrathionate in the absence of yeast extract were compared by electrophoresis with cells from Sulfolobus (Birch Coppice) all 10 of the major bands were in identical positions (Fig.47). S. acidocaldarius (from J. Brierley) possessed 5 major bands (<) in common with Sulfolobus (Lake Myvam) and the same 5 common bands were also present in S. brierleyi (+). Sulfolobus B6-2 possessed 5 protein bands in common with Sulfolobus (Lake Myvam) (—+) two of which were found in all the tetrathionate-grown cultures of Sulfolobus.

A comparison of pyrite-grown cells (Fig.48) from S. brierleyi and Sulfolobus (Lake Myvam)(see Section 7.4) showed that of the 9 marked protein bands of Sulfolobus (Lake Myvam) (—) only one was present in S. brierleyi (<). In contrast the 9 bands of Sulfolobus (Lake Myvam) were also present in identical

Fig. 46 Electrophoretic protein patterns of whole cell lysates from S. solfataricus (A,E), S. acidocaldarius (B,D) and Sulfolobus (Lake Myvum) (C) grown on yeast extract.

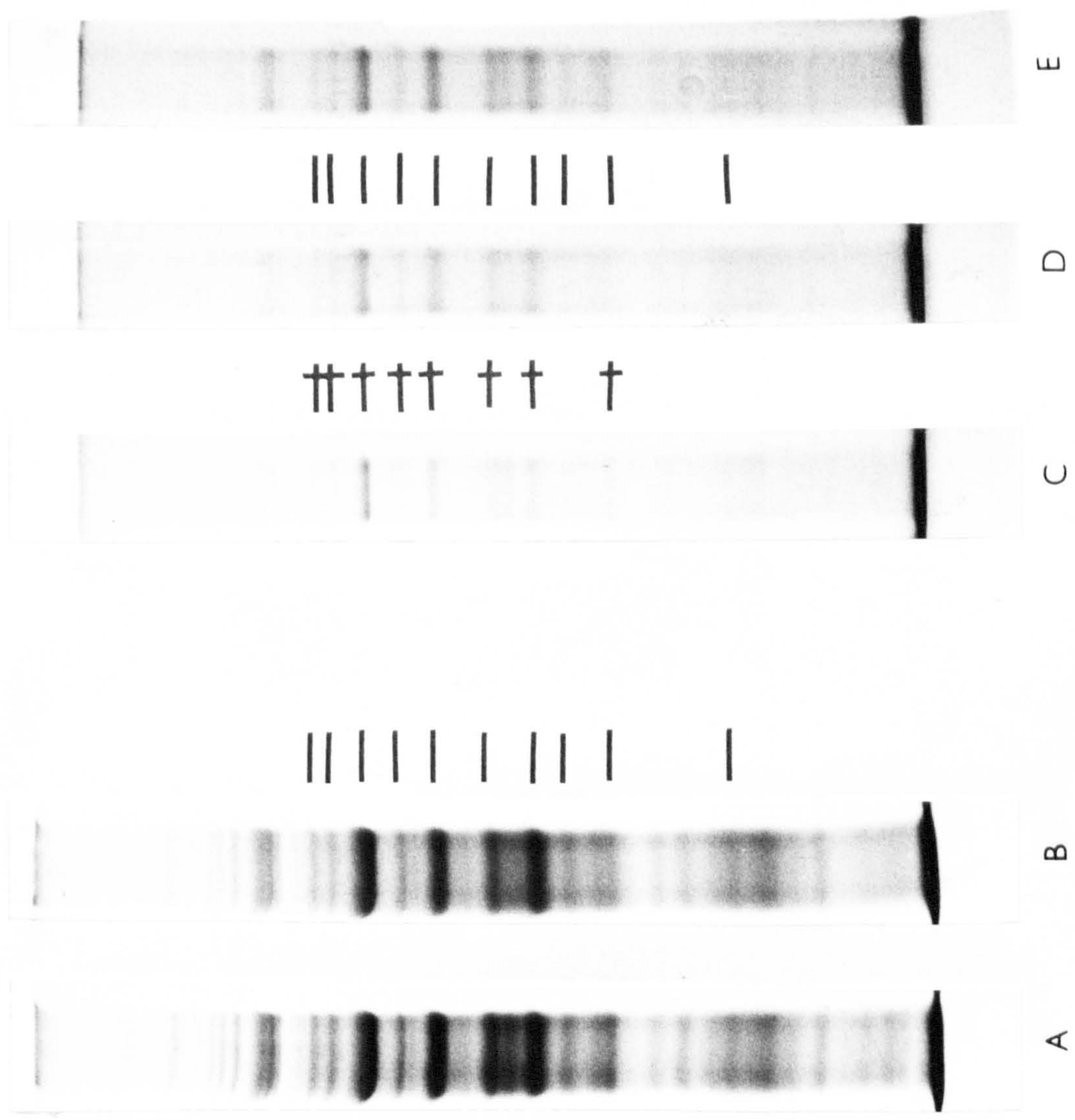


Fig. 47 Electrophoretic protein patterns of whole cell lysates of Sulfolobus (Lake Myvum) grown autotrophically on pyrite (A), sulphur (B) and tetrathionate (C & D) and of (all grown on tetrathionate) Sulfolobus (Birch Coppice) (E), S. brierleyi (F), Sulfolobus B6-2 (G) and S. acidocaldarius (from J. Brierley) (H).

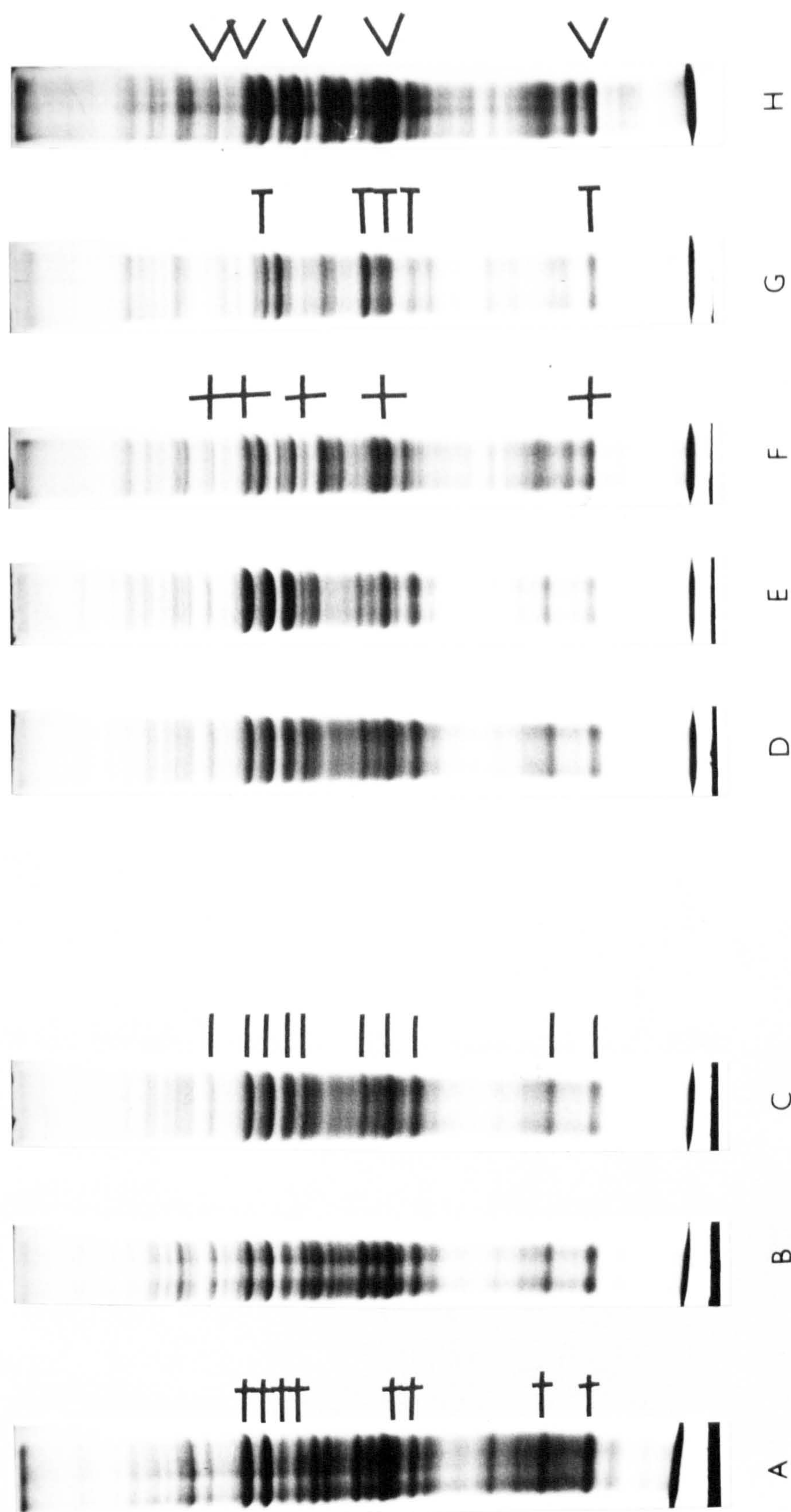
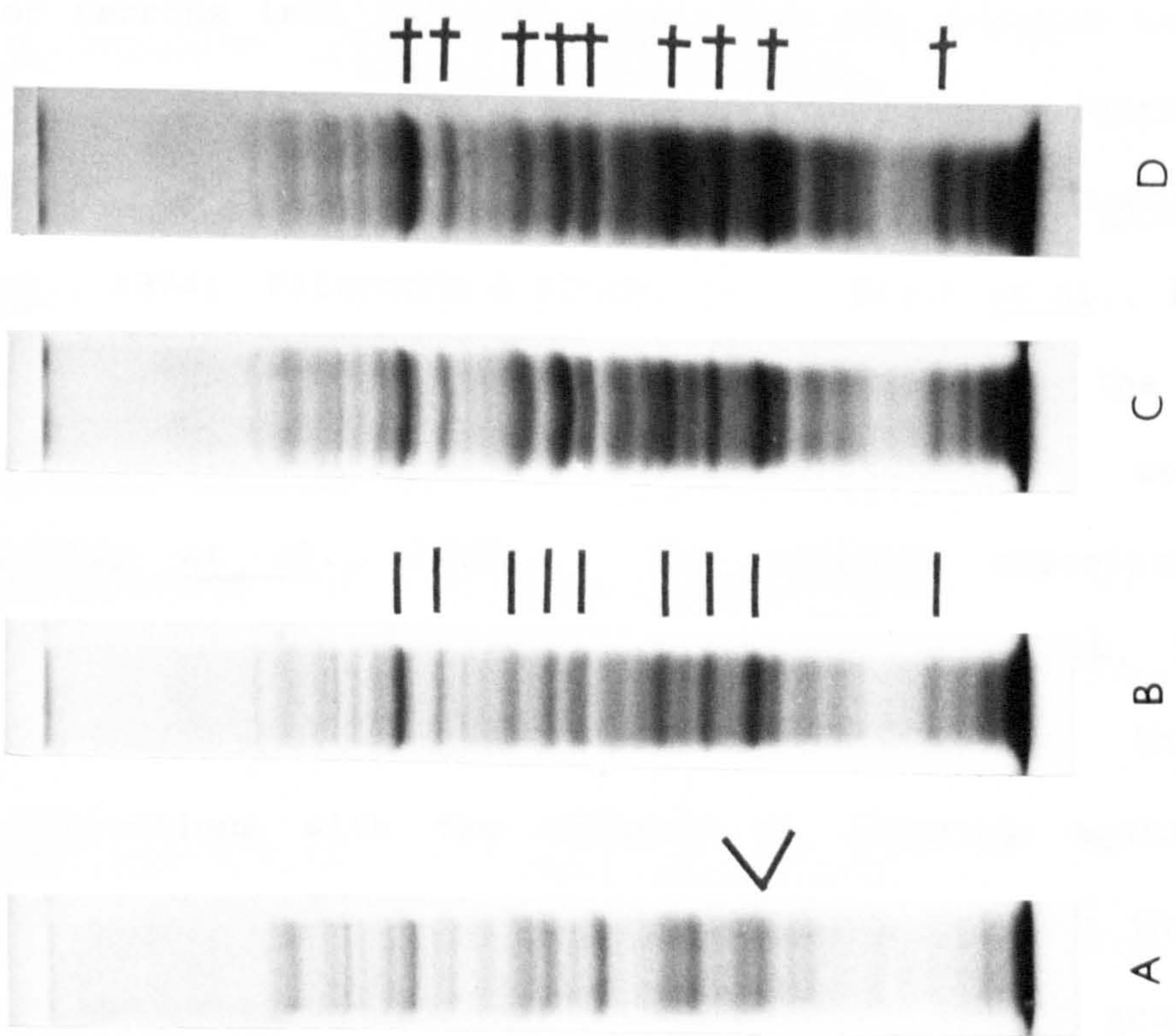


Fig. 48 Electrophoretic protein patterns of whole cell lysates S. brierleyi (A) and Sulfolobus (Lake Myvam) (B) grown on pyrite in the presence of yeast extract and of Sulfolobus (Lake Myvam) (C) and Sulfolobus (Birch Coppice) (D) grown autotrophically on pyrite.



positions in pyrite-grown cultures of Sulfolobus (Birch Coppice).

There was no observable difference in the electrophoregram of Sulfolobus (Lake Myvam) grown autotrophically on pyrite and that of it grown on pyrite in the presence of yeast extract (Fig.48).

6.8 DISCUSSION

The increase in culture pH which occurred when S.acidocaldarius 98-3 and S. solfataricus were grown in the presence of elemental sulphur and yeast extract indicates that they were not growing on the sulphur but on the yeast extract alone. The inability of these strains to oxidize either sulphur or ferrous iron indicates that they are obligate heterotrophs. This is in contrast to the sulphur and iron oxidation which has been reported for natural populations of Sulfolobus (Mosser et al., 1974; Fliermans & Brock, 1972; Brock et al., 1976) as well as numerous accounts of sulphur oxidation by the type strain S.acidocaldarius 98-3 (Shivvers & Brock, 1973; Weiss, 1973; Zillig et al., 1980). The original descriptions of S. acidocaldarius as mixotrophic (Shivvers & Brock, 1973) should possibly be reconsidered in the light of these present observations with the emphasis on ensuring against a mixed population of autotrophs and heterotrophs giving the appearance of a mixotrophic culture. The doubling time for growth on yeast extract by S. acidocaldarius 98-3 was slightly higher than that previously reported (Brock et al., 1972) but rates have been shown to be strain dependent.

When the enrichment culture of Sulfolobus (Lake Myvam) was grown on yeast extract over several sub-cultures a 'heterotrophic

-type' of organism, similar to S. acidocaldarius was selected. This would account for its subsequent inability to revert back to oxidizing ferrous iron. Even though the two different mixed populations of Sulfolobus (Lake Myvam) were morphologically indistinguishable, the electrophoretic data confirms that the heterotrophic population of Sulfolobus (Lake Myvam) was similar to S. acidocaldarius 98-3 and S. solfataricus. It is possible that S. acidocaldarius 98-3, while being kept in the presence of yeast extract, has undergone the same selection procedure as occurred with Sulfolobus (Lake Myvam) in the short term, and that the organisms responsible for sulphur oxidation in this particular culture have been lost from the population.

The observation that yeast extract is required by S. brierleyi for sustained growth on ferrous iron confirms previous reports that yeast extract enhanced growth (Brierley & Brierley, 1973). However, even in the presence of yeast extract the doubling times for growth on ferrous iron were slow when compared with the mesophile T. ferrooxidans (Norris & Kelly, 1982) and the moderately thermophilic iron-oxidizing bacteria (see Section 3.4).

The rate of iron oxidation by Sulfolobus (Lake Myvam) was similar to that observed using Sulfolobus strain 36-1 (Brock, 1978). In contrast to the moderately thermophilic iron-oxidizing bacteria, where a several-fold increase in iron oxidation rate in the presence of yeast extract was often observed, only a slight increase in rate occurred in the presence of yeast extract.

The growth rate of Sulfolobus (Lake Myvam) on sulphur was similar to that observed by the moderately thermophilic thiobacillus-type bacterium LM7 isolated from the same site (Fig.32) but faster than that observed by the moderately thermophilic iron-oxidizer LM2. The rate of sulphur oxidation was more rapid than previously reported for some Sulfolobus strains (Shivvers & Brock, 1973) but earlier work involved static non-aerated cultures.

Sulfolobus B6-2 was the only strain capable of growing on sulphur but not on ferrous iron. Although detailed physiological characteristics were not determined, the electrophoretic comparison (Fig.47) confirms that it is probably a different species of Sulfolobus to either of the other sulphur-oxidizers, Sulfolobus (Lake Myvam) and S. brierleyi.

S. brierleyi appeared during all observations to be identical to S. acidocaldarius (from J. Brierley) and it is probable that these two cultures are identical and both S.brierleyi.

During all basic physiological and electrophoretic studies Sulfolobus (Lake Myvam) appeared identical to Sulfolobus (Birch Coppice) but recent work has indicated that there are probable differences in their tolerance to certain metals. Some differences might be expected in view of the different characters of the isolation sites.

All observations suggest that at present, based on the physiological characteristics and the electrophoregram comparisons that there are 4 'groups' of Sulfolobus-like

organisms (Table 3), Group 1, characterised by S. acidocaldarius 98-3 are obligate heterotrophs. Group 2, characterised by Sulfolobus B6-2, can maintain growth on sulphur but not on ferrous iron. Group 3, characterised by Sulfolobus (Lake Myvam), can maintain autotrophic growth on iron and sulphur although whether such organisms will also grow on organic substrates has not been proven. Group 4, characterised by S. brierleyi, will grow on sulphur and ferrous iron but autotrophic growth on iron cannot be sustained even in the presence of a reduced sulphur source.

This study has outlined some of the problems when comparing the present Sulfolobus strains and indicated that S. acidocaldarius 98-3 in one collection might not be identical to that in another collection. An effective method of obtaining pure cultures must be found before further comparative studies can be undertaken. Detailed evolutionary and taxonomic studies and DNA-DNA homology on pure cultures need to be performed to confirm that the new isolates, Sulfolobus (Lake Myvam) and Sulfolobus (Birch Coppice), are a different species from S. acidocaldarius 98-3, S. solfataricus and S. brierleyi.

TABLE 3 A COMPARISON OF SUBSTRATE UTILIZATION BY SULFOLOBUS STRAINS

ORGANISM	SUBSTRATE			GROUP
	Yeast Extract	Fe ²⁺	S ⁰	
<u>S. acidocaldarius</u> 98-3	+	-	-	1
<u>S. solfataricus</u>	+	-	-	
<u>Sulfolobus</u> (Lake Myvam) (grown on yeast extract)	+	-	-	
<u>Sulfolobus</u> B6-2	N.D.	-	+	2
<u>Sulfolobus</u> (Lake Myvam)	N.D.	+	+	3
<u>Sulfolobus</u> (Birch Coppice)	N.D.	+	+	
<u>S. brierleyi</u>	N.D.	+	+	4
<u>S. acidocaldarius</u> (from J. Brierley)	N.D.	+	+	

P A R T 7

GROWTH OF THERMOPHILES ON MINERAL SULPHIDES

7.1 INTRODUCTION

The ability of the moderately thermophilic, iron-oxidizing bacteria to solubilize mineral sulphides at 50°C has been demonstrated using a range of metal sulphides (Norris & Kelly, 1978; LeRoux et al., 1977; Mehta & Murr, 1982) including copper, nickel and uranium ores. They have also been described as capable of solubilizing the pyritic element in coal (Murr & Mehta, 1982).

Thermophile TH1 was able to oxidize pyrite up to 55°C at which temperature it was reported to be unable to oxidize ferrous iron (Brierley & LeRoux, 1977). The maximum rate of pyrite oxidation by resting cell suspensions of TH1 occurred at a pH of 2.6 with little oxidation at either pH 1.1 or 3.5. The rate of oxidation also increased with increasing pulp density up to a maximum of 100g of pyrite per litre, the maximum tested (Brierley & LeRoux, 1977).

The yield of copper from the dissolution of copper concentrates by moderately thermophilic bacteria at 30°C has been demonstrated to be similar to yields obtained using mesophilic mineral-oxidizing bacteria (Norris et al., 1980) at the same temperature but at higher temperatures (50°C), a greater yield occurred. However, all previous demonstrations of bacterially-assisted mineral dissolution at 50°C have indicated a requirement for organic nutrients (Brierley & LeRoux, 1977; Norris et al., 1980) with the level of supplement required sometimes varying between organisms. Thermophile TH1 has been shown to require a

higher concentration of yeast extract than thermophile TH3 to produce an equivalent extent of growth-linked iron dissolution from pyrite (Norris et al., 1980).

When thermophiles TH1 and TH3 were grown on pyrite the pH of the medium fell suggesting that sulphur oxidation was occurring (Norris et al., 1980). However, when grown on chalcopyrite the pH of the medium containing TH3 produced a greater rise in pH than shown by TH1 (Norris et al., 1980) indicating a difference in the extent of sulphur oxidation by the two bacteria on this substrate. Variations in the tolerance of moderately thermophilic iron-oxidizing bacteria to heavy metals have been shown to exist between the strains (J.A. Brierley, personal communication). These variations in growth characteristics and different responses to some growth conditions, such as organic nutrient levels and toxic metals, cannot yet be fully understood because there has previously been very little fundamental study of the interaction of moderately thermophilic mineral-oxidizing bacteria with mineral sulphides.

Among the extreme thermophiles, Sulfolobus brierleyi has been the most extensively used bacterium in demonstrations of mineral leaching. It has been shown to produce 90% extraction of copper from certain ores using a 1% (w/v) pulp density at 60°C during shake flask leaching experiments (C.L. Brierley, 1977). A higher level of extraction than obtained with T. ferrooxidans has also been demonstrated during column leaching (C.L. Brierley, 1980). However, the extent of the leaching was dependent on the

mineralogy of the sample used and the addition of yeast extract and ferrous iron to the medium gave improvements in leaching rates with some copper ores (Brierley & Brierley, 1978).

Leaching of molybdenite by S. brierleyi at 60°C resulted in some molybdenum extraction (Brierley & Murr, 1973; C.L. Brierley 1977). The organism was tolerant to high levels of molybdenum with 1,000 ppm causing a reduction in the sulphur oxidation rate compared with the maximum tolerance reported for other chemoautotrophic bacteria of 5 to 90 ppm (Tuovinen et al., 1971b).

Investigations using the electron microscope revealed that S. brierleyi attached directly to mineral surfaces of some ores (Brierley & Murr, 1973; Murr & Berry, 1976).

S. acidocaldarius has recently been used during experiments on the desulphurization of coal (Detz & Barvinchak, 1979; Kargi & Robinson, 1982a,b). The rate of sulphur removal was shown to be dependent on the sulphur content of the coal and the total external surface area (Kargi & Robinson, 1982b). The addition of yeast extract, peptone and ferric iron showed an adverse effect on sulphur removal from coal (Kargi & Robinson, 1982a). An economic study has compared potential coal desulphurization processes in lagoons using T. ferrooxidans, S. acidocaldarius and S. brierleyi and concluded that costs per ton would be similar (Detz & Barvinchak, 1979) but that the high temperature leaching would be more suitable where available land was at a premium as less space was required for similar throughput rates. Bacterially-assisted coal desulphurization was shown to be potentially less expensive than

some chemical oxidation processes (Detz & Barvinchak, 1979).

The aim of initial work on moderately thermophilic bacterial mineral oxidation was to evaluate the organic nutritional requirements during growth of the bacteria. Variations had already been shown to exist between moderately thermophilic bacteria and as new isolates were collected, comparisons of their pyrite oxidizing ability were made. After the demonstration of their chemolithoautotrophic growth during ferrous iron oxidation (see Section 3.4), the capacity for autotrophic growth on mineral sulphides was investigated. Similarly, the organic nutritional requirements during mineral oxidation by the newly isolated extremely thermophilic bacteria was compared with existing isolates and their capacity for autotrophic mineral oxidation was assessed.

7.2 MODERATELY THERMOPHILIC BACTERIA: PYRITE OXIDATION

The oxidation of pyrite during growth of thermophile TH1 with a range of medium supplements at 50°C was determined. When the medium was supplemented with yeast extract ($0.1 - 1.0\text{gl}^{-1}$), the initial rate of iron solubilization was independent of the concentration of yeast extract (Fig.49). However, the faster rate of solubilization continued to give the highest release of iron into solution using 0.5g yeast extract per litre. When the pyrite medium was supplemented with glutathione ($0.1 - 1.0\text{gl}^{-1}$) the highest level of iron solubilization occurred using the lowest concentration of glutathione (0.1gl^{-1})(Fig.50). At 400 hours the level of iron released from the pyrite was only 2.2gl^{-1}

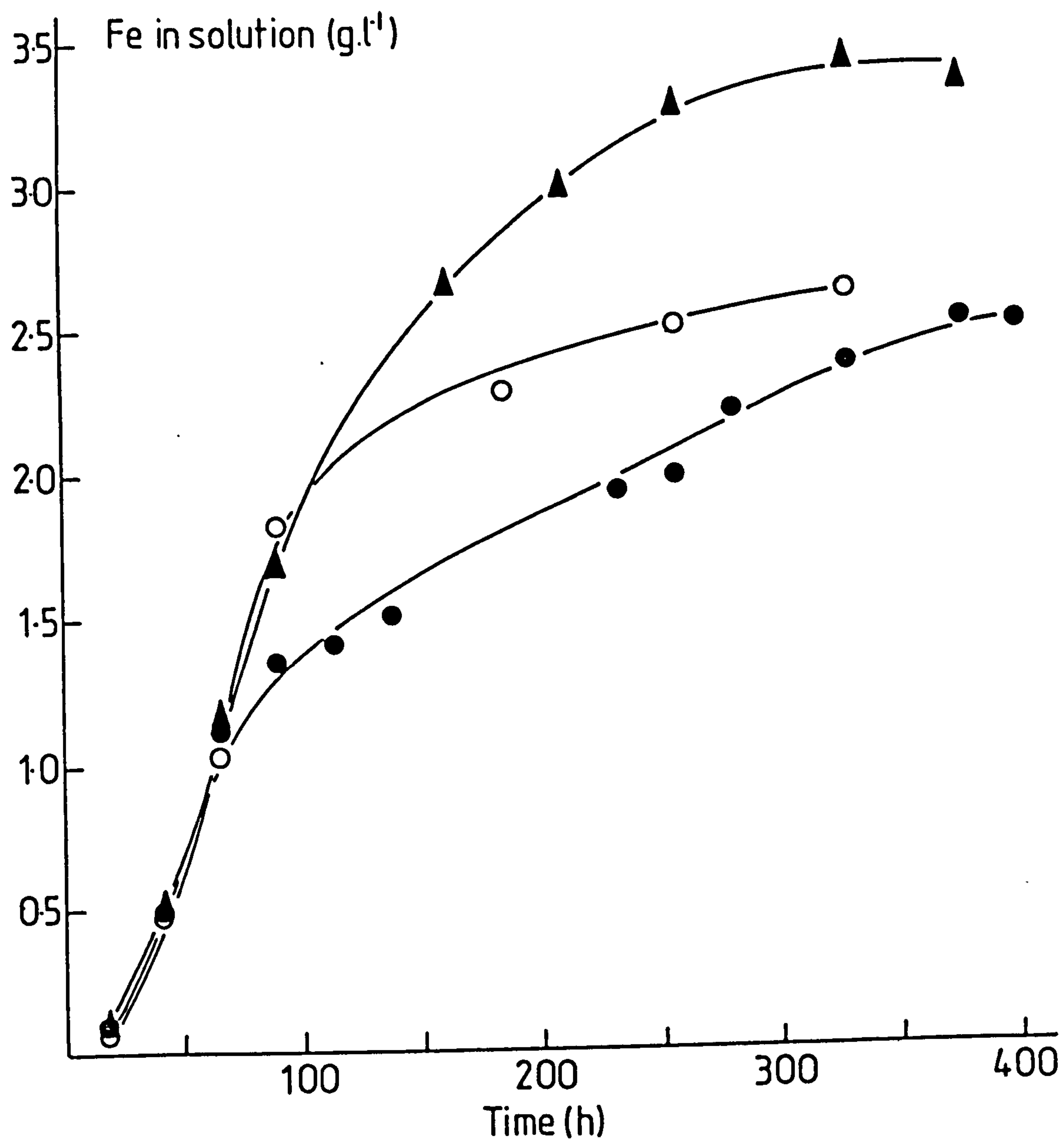


Fig. 49 The solubilization of iron from pyrite (10 g.l^{-1}) at 50°C by thermophile TH1. The medium was supplemented with yeast extract to initial concentrations of (g.l^{-1}) of 0.1 (●), 0.5 (▲), and 1.0 (○).

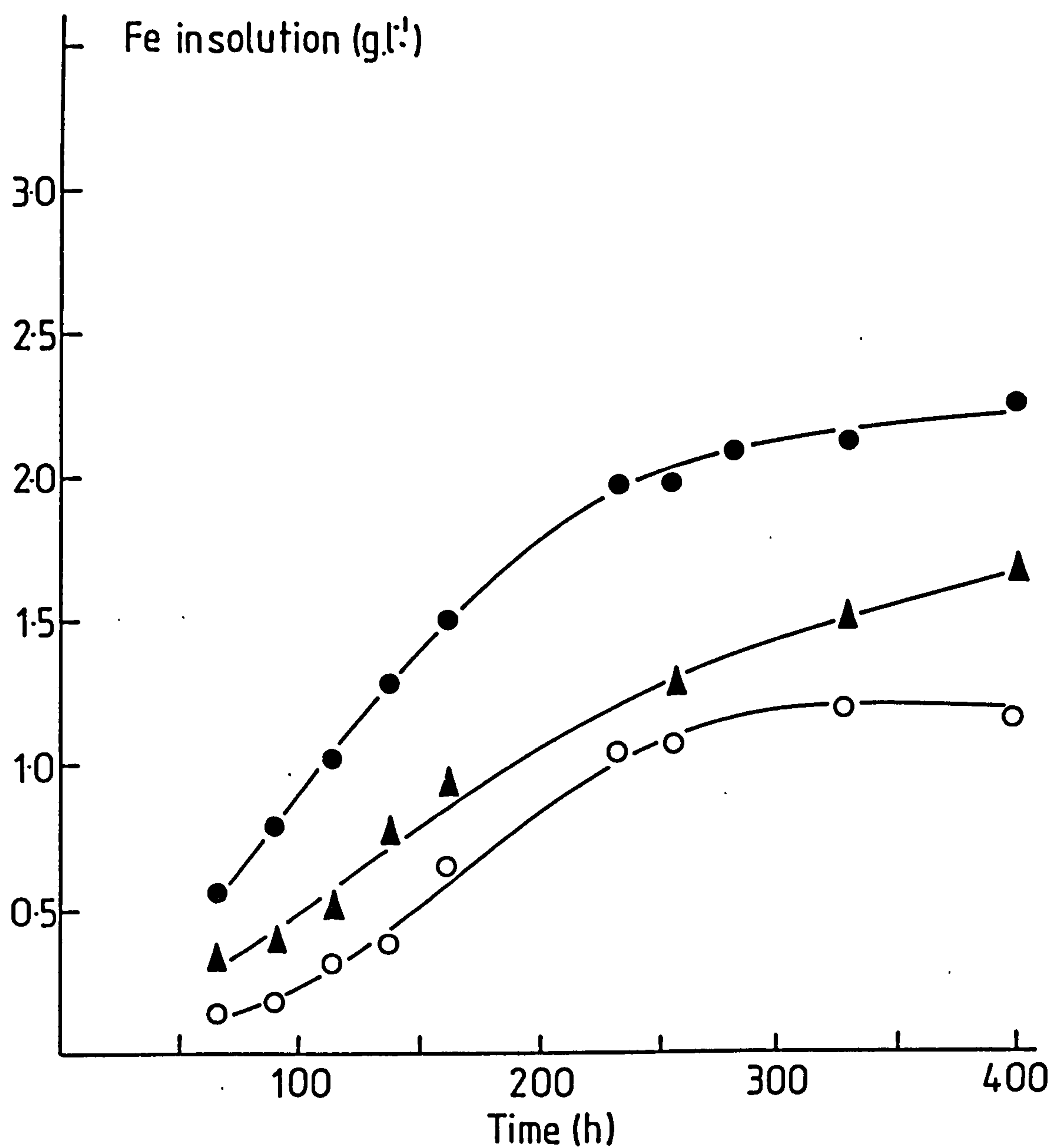


Fig. 50 The solubilization of iron from pyrite (10 g.l^{-1}) at 50°C by thermophile TH1. The medium was supplemented with glutathione to initial concentrations (g.l^{-1}) of 0.1 (●), 0.5 (▲) and 1.0 (○).

compared with an iron release at 400 hours of 3.4gl^{-1} when yeast extract (0.5gl^{-1}) was used. When the pyrite medium was supplemented with glucose ($0.1 - 1.0\text{gl}^{-1}$), the iron released into solution was independent of the concentration of glucose used (Fig.51). At 400 hours the level of iron in solution was 2.2gl^{-1} but the rate of release was considerably slower than when the medium was supplemented with yeast extract. The rate of iron release had not begun to level out but appeared to be still increasing with time.

A comparison of pyrite oxidation was made between thermophile TH1 and the 50°C enrichment cultures from the INCO Ltd. mines, Kingsbury and Alvecote. When a low concentration of pyrite (10gl^{-1}) was used with a low level of yeast extract (0.2gl^{-1}) all cultures solubilized all the available iron and the final culture pH was similar (Fig.52). However, the rate of iron release was significantly slower using the Alvecote 50°C enrichment culture when compared with the other cultures (Table 4).

When the amount of available pyrite was increased (50gl^{-1}) and the yeast extract concentration unchanged, incomplete pyrite oxidation was observed and variations occurred between cultures in the degree of solubilization of iron (Fig.53). Approximately 50% of the available iron was solubilized by the Kingsbury 50°C enrichment culture which also had the faster rate of iron solubilization. A similar rate occurred when using the INCO Ltd. mine enrichment culture but slightly less iron was solubilized at

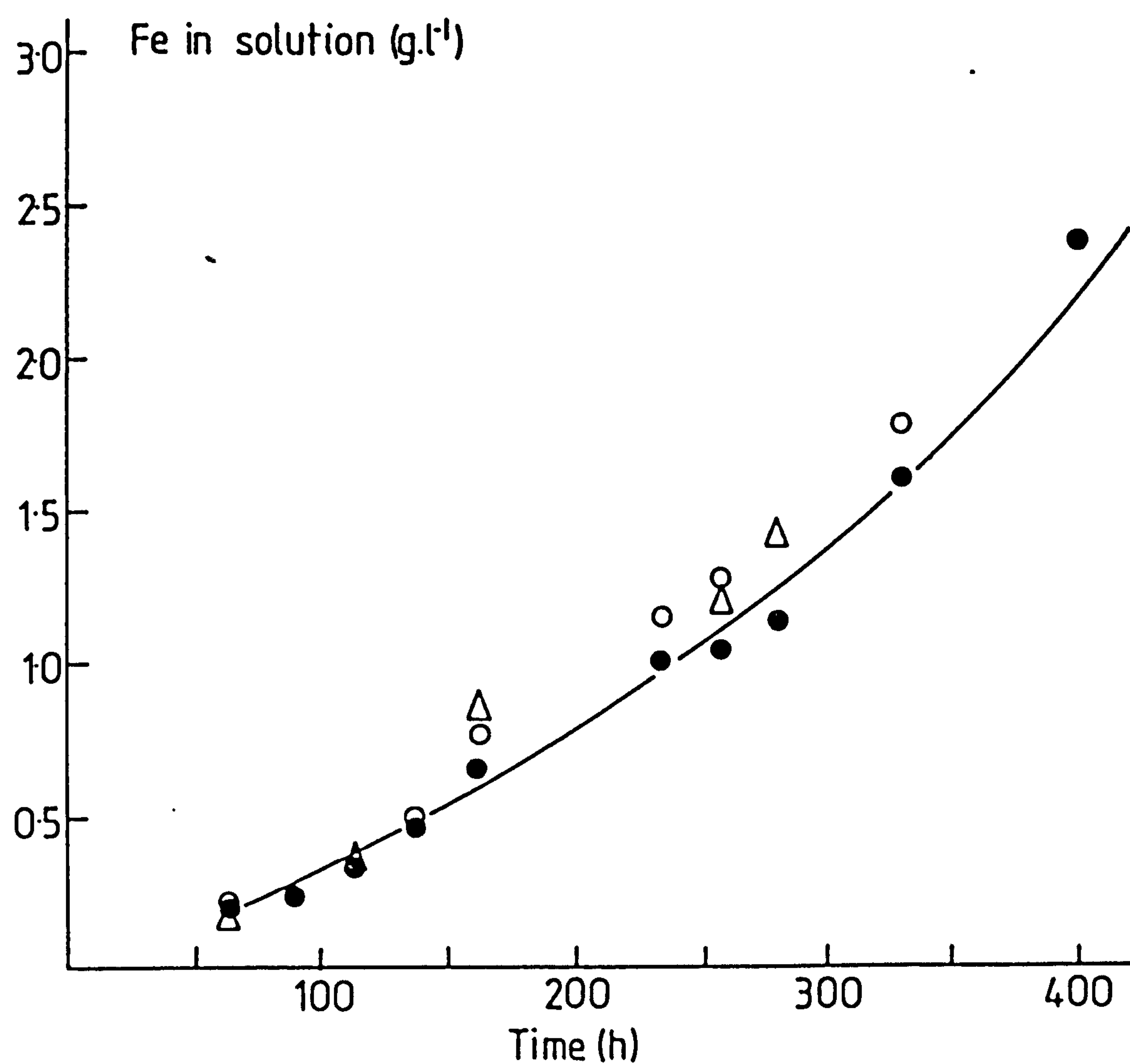


Fig. 51 The solubilization of iron from pyrite (10 g.l^{-1}) at 50°C by thermophile TH1. The medium was supplemented with glucose to initial concentrations (g.l^{-1}) of 0.1 (●), 0.5 (Δ) and 1.0 (○).

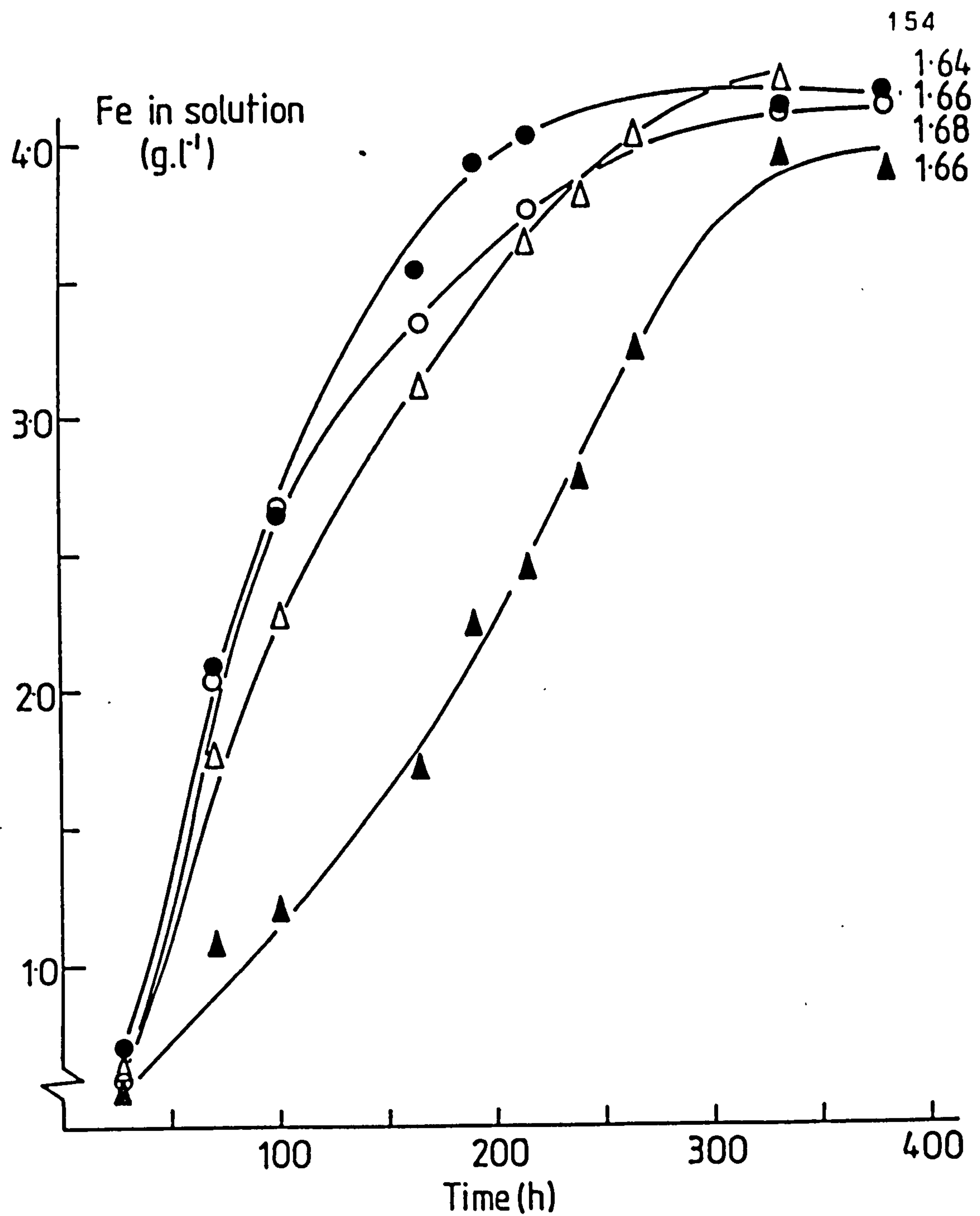


Fig. 52 The solubilization of iron from pyrite (10g.l^{-1}) at 50°C by thermophile TH1 (○) and by the INCO (△), Kingsbury (●) and Alvecote (▲) 50°C enrichment cultures. The medium was supplemented with yeast extract (0.2g.l^{-1}) and the final medium pH values are indicated adjacent to the key symbols.

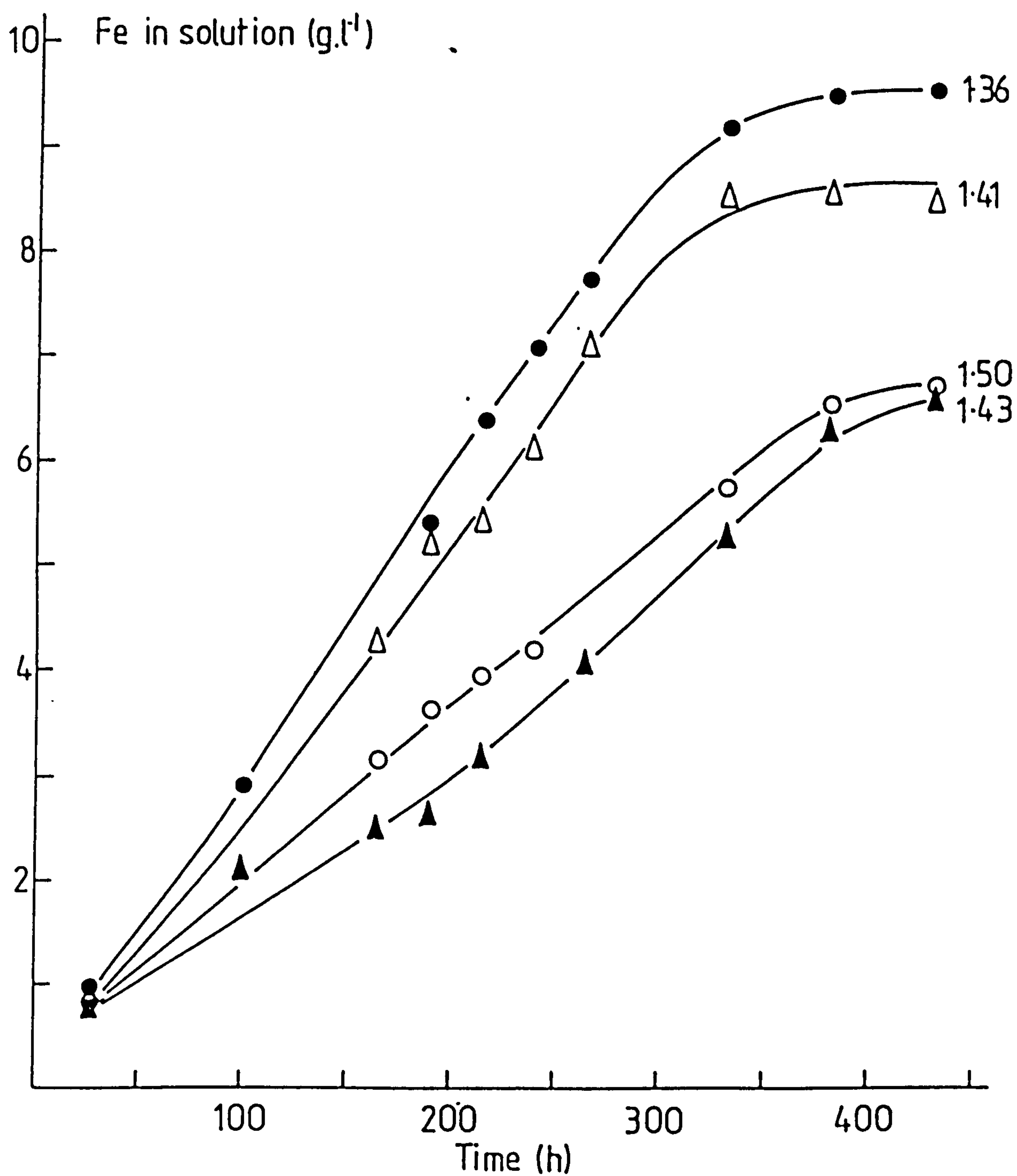


Fig. 53 The solubilization of iron from pyrite (50g.l^{-1}) at 50°C by thermophile TH1 (○) and by the INCO (Δ), Kingsbury (●) and Alvecote (▲) 50°C enrichment cultures. The medium was supplemented with yeast extract (0.2g.l^{-1}) and the final medium pH values are indicated.

400 hours. TH1 and the Alvecote 50°C enrichment culture both showed poorer rates of iron solubilization (Table 4) and only approximately 30% of the available iron was solubilized. The final pH of all cultures was lower than when pyrite at 10gl^{-1} was used.

To investigate whether the organic nutrient level was limiting pyrite oxidation the yeast extract concentration was increased to 0.5gl^{-1} . All cultures showed a lower level of iron in solution at 400 hours than when the yeast extract concentration was 0.2gl^{-1} (Fig.54).

TH1 and the Kingsbury 50°C enrichment culture were used to investigate the effect that the increasing acidity which occurs during pyrite oxidation had on bacterial growth (Fig.55). At 265 hours, the pH of the Kingsbury 50°C enrichment culture and TH1 were 1.15 and 1.25 respectively and the rate of pyrite dissolution was decreasing. The resuspension of bacteria and pyrite into fresh medium at the initial pH of 2.0 resulted in further pyrite dissolution but the resuspension in fresh medium at pH 1.2 did not.

Following the demonstration of autotrophic iron oxidation (see Section 3.4) by the moderately thermophilic bacteria autotrophic growth on pyrite was established. Autotrophic growth by the Birch Coppice isolate was maintained through several serial sub-cultures with no reduction in the rate or extent of pyrite oxidation. The doubling times for iron solubilization (Fig.56) were taken from the period of most rapid iron release at

TABLE 4 A COMPARISON OF RATES OF IRON SOLUBILIZATION FROM PYRITE
UNDER DIFFERENT CONDITIONS BY MODERATELY THERMOPHILIC BACTERIA

CULTURE	Rate of Iron Solubilization (mg h^{-1})		
	$\text{FeS}_2(10\text{gl}^{-1})$	$\text{FeS}_2(50\text{gl}^{-1})$	$\text{FeS}_2(50\text{gl}^{-1})$
	Y.E.(0.2gl^{-1})	Y.E.(0.2gl^{-1})	Y.E.(0.5gl^{-1})
TH1	26	16	N.D.
INCO Ltd. mine enrichment culture	20	28	N.D.
Kingsbury 50°C enrichment culture	28	30	26
Alvecote 50°C enrichment culture	13	17	15

Y.E. = yeast extract

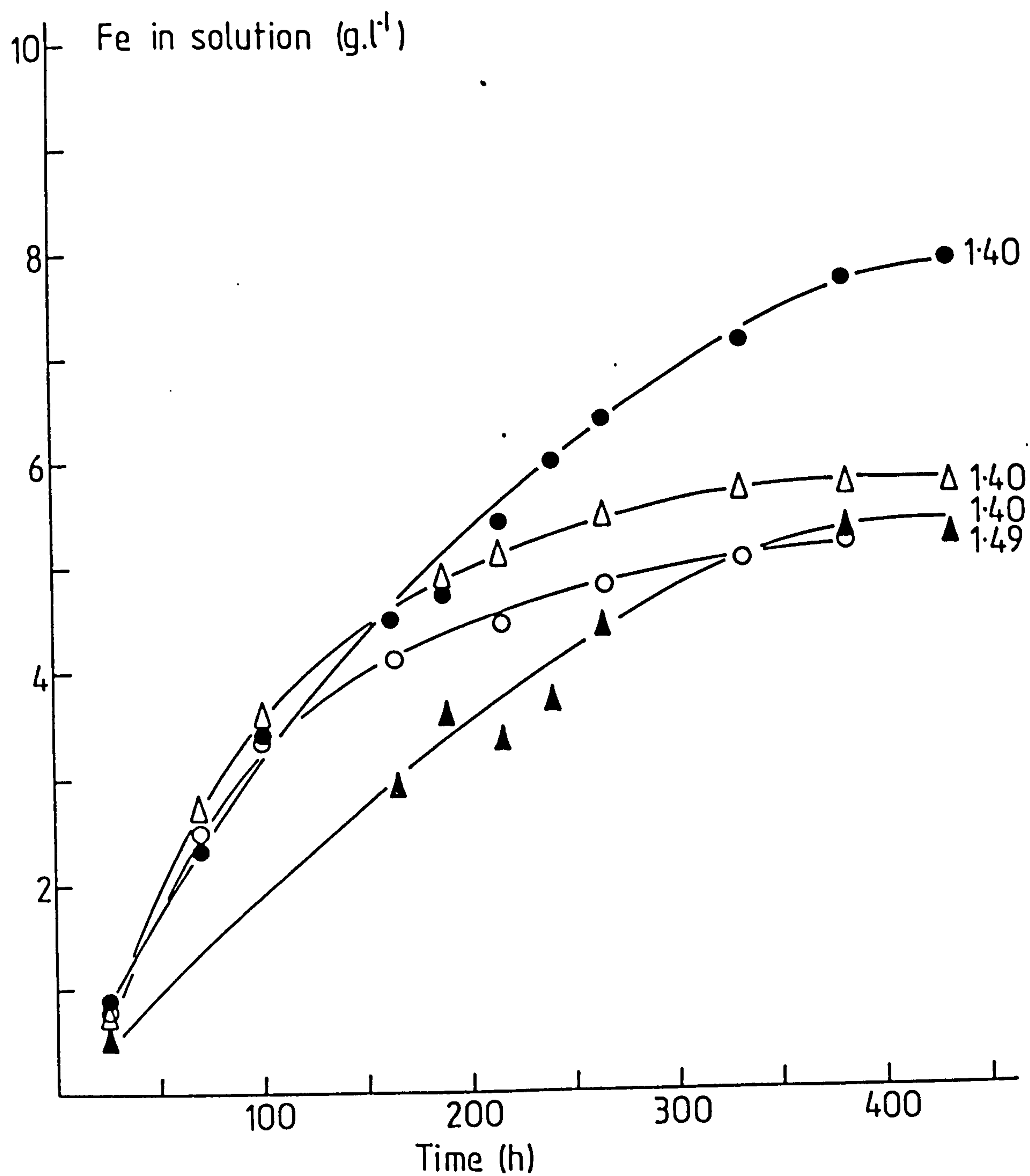


Fig. 54 The solubilization of iron from pyrite (50g.l^{-1}) at 50°C by thermophile TH1 (○) and by the INCO (Δ), Kingsbury (●) and Alvecote (▲) 50°C enrichment cultures. The medium was supplemented with yeast extract (0.5g.l^{-1}) and the final medium pH values are indicated.

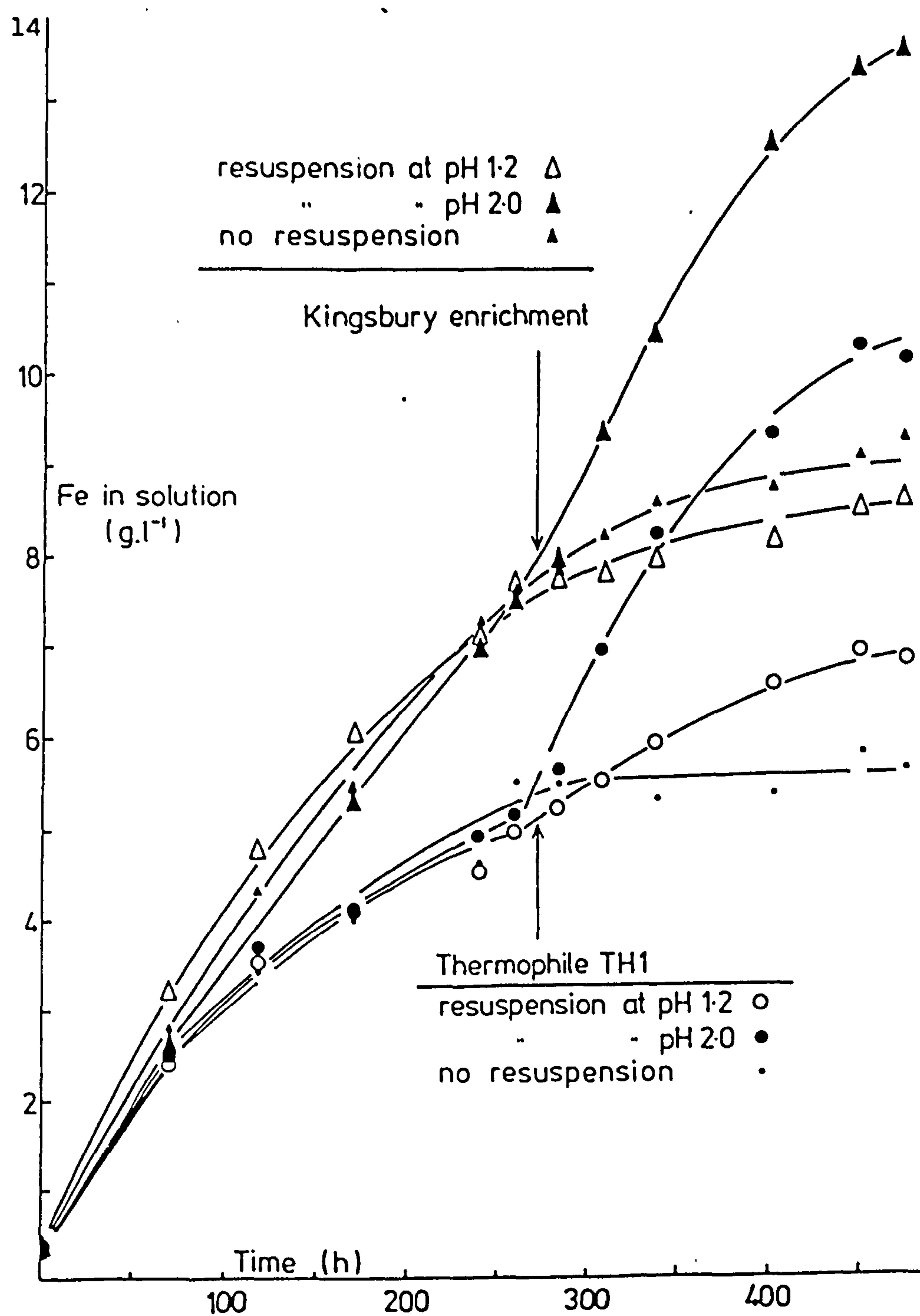


Fig. 55 The solubilization of iron from pyrite (50g.l^{-1}) at 50°C by thermophile TH1 and the Kingsbury 50°C enrichment culture. The medium was supplemented with yeast extract (0.2g.l^{-1}).

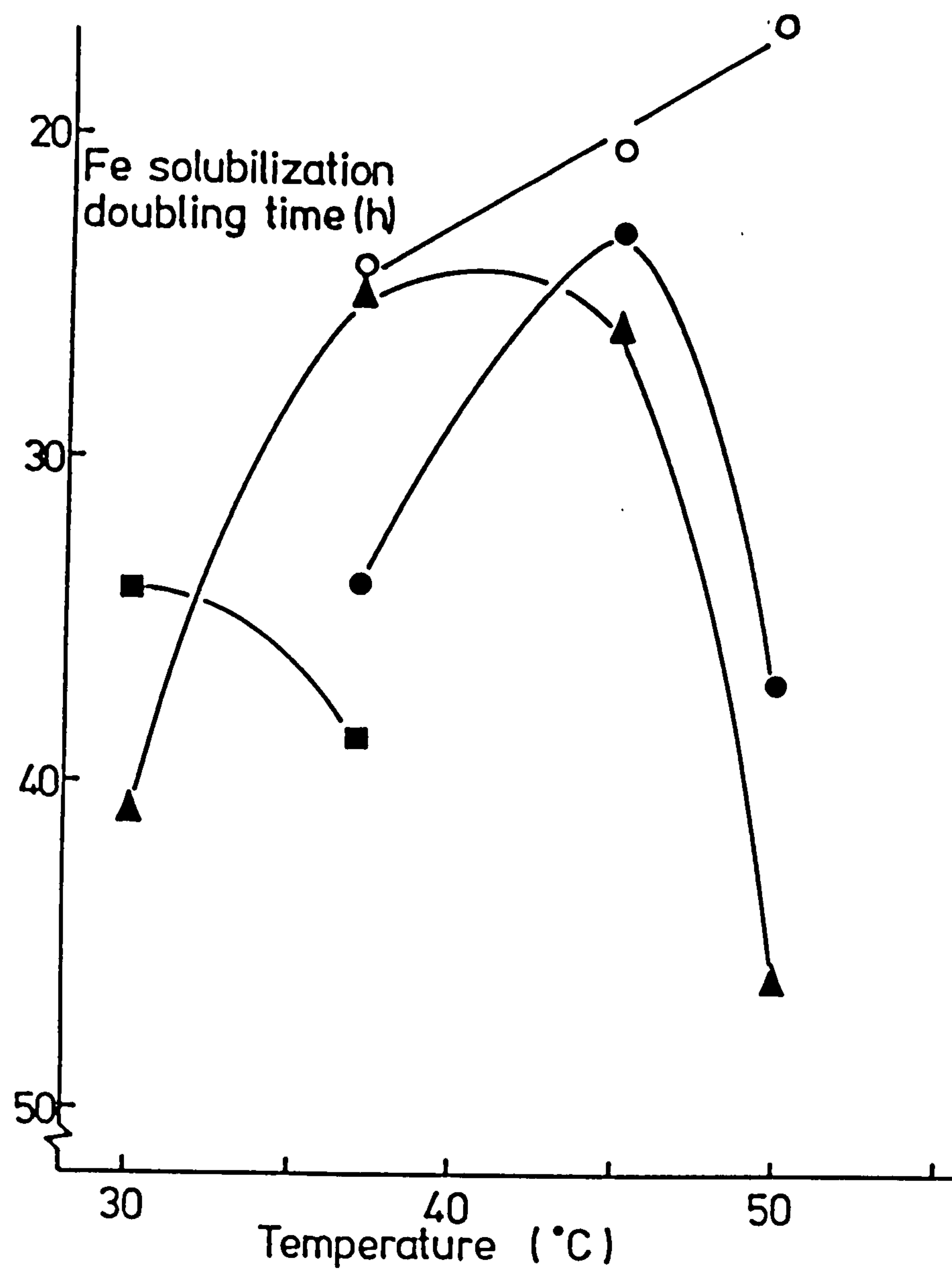


Fig. 56 The effect of temperature on the dissolution of pyrite (10 g.l^{-1}) during autotrophic growth of the Lake Myvam 40°C enrichment culture (▲), the Birch Coppice isolate (●) and *T. ferrooxidans* (■) and during growth of the Birch Coppice isolate (○) in the presence of yeast extract (0.2 g.l^{-1}).

each temperature. The maximum rate of pyrite dissolution by the Birch Coppice isolate during autotrophic growth was at 45°C, with a reduced rate at 50°C. However, in the presence of yeast extract the rate of pyrite dissolution was slightly higher at 45°C and 50°C with the fastest rate being obtained at 55°C.

T. ferrooxidans did not grow at 40°C and had a faster rate of pyrite dissolution at 30°C than 37°C. The Lake Myvam 40°C enrichment culture showed a wide temperature range for pyrite dissolution with the maximum rate being obtained between 37°C–45°C.

7.3 MODERATELY THERMOPHILIC BACTERIA: CHALCOPYRITE OXIDATION

In contrast to pyrite oxidation by the Birch Coppice isolate the rate of chalcopryrite (10gl^{-1}) dissolution during autotrophic growth was only slightly inhibited at 55°C (Fig.57) compared to 50°C but at 60°C was severely inhibited. In the presence of yeast extract the rate of copper release was still fastest at 50°C with severe inhibition at 60°C (Fig.58). A higher yield of copper might have been expected at 50°C than at 46°C in view of the data from autotrophic growth and in view of the following results with other strains.

The mesophile T. ferrooxidans showed extensive chalcopryrite oxidation and copper release at 37°C but not at 45°C (Fig.59). The initially higher level of copper present in the inoculated flasks compared to the sterile control was due to rapid oxidation of some of the copper by soluble ferric iron in the pyrite grown inocula. The Lake Myvam 40°C-enrichment culture showed a similar

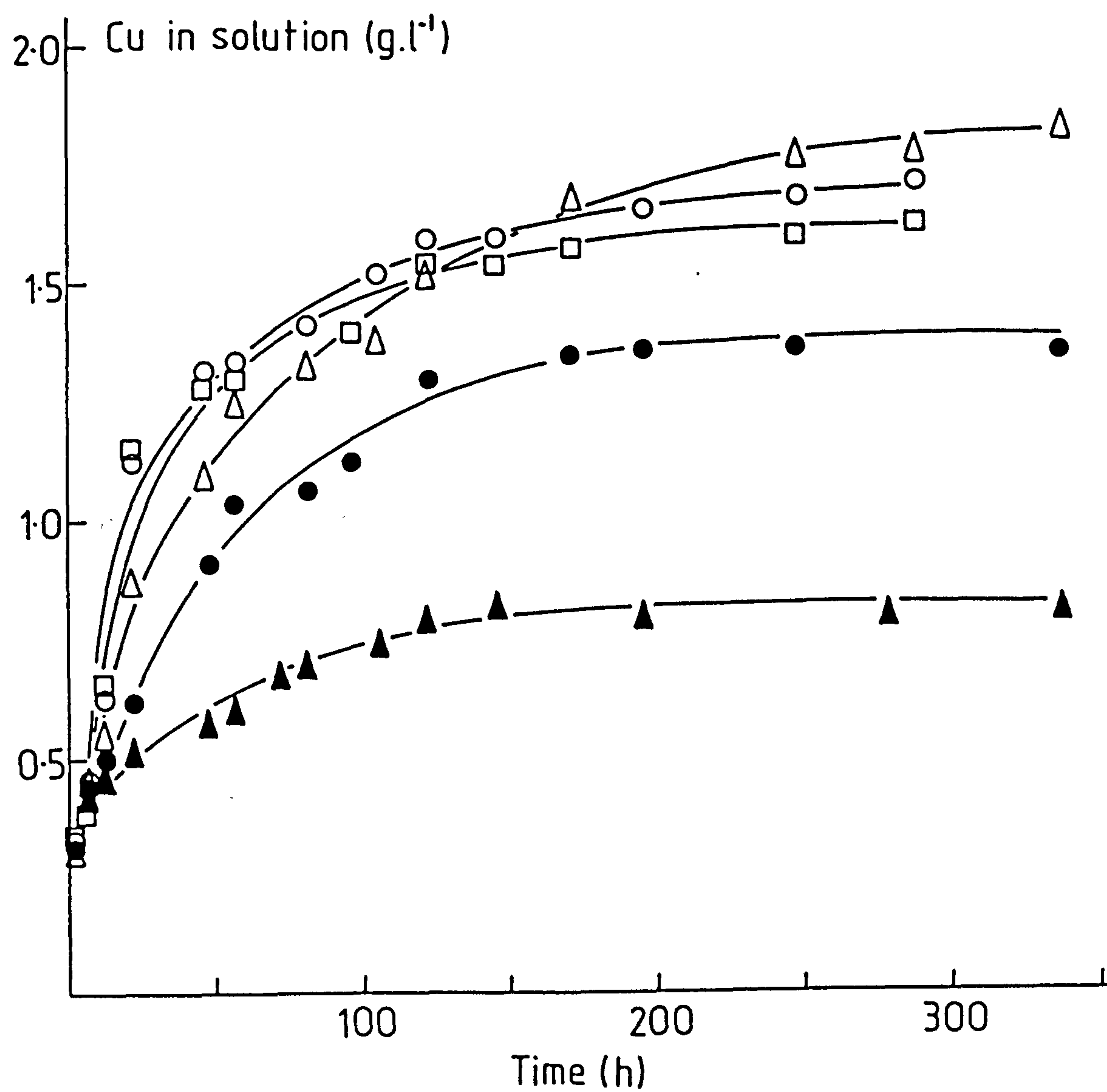


Fig. 57 Copper solubilization from chalcopryrite (10g.l^{-1}) during autotrophic growth of the moderately thermophilic Birch Coppice isolate at 38°C (●), 46°C (□), 50°C (○), 55°C (△) and 60°C (▲).

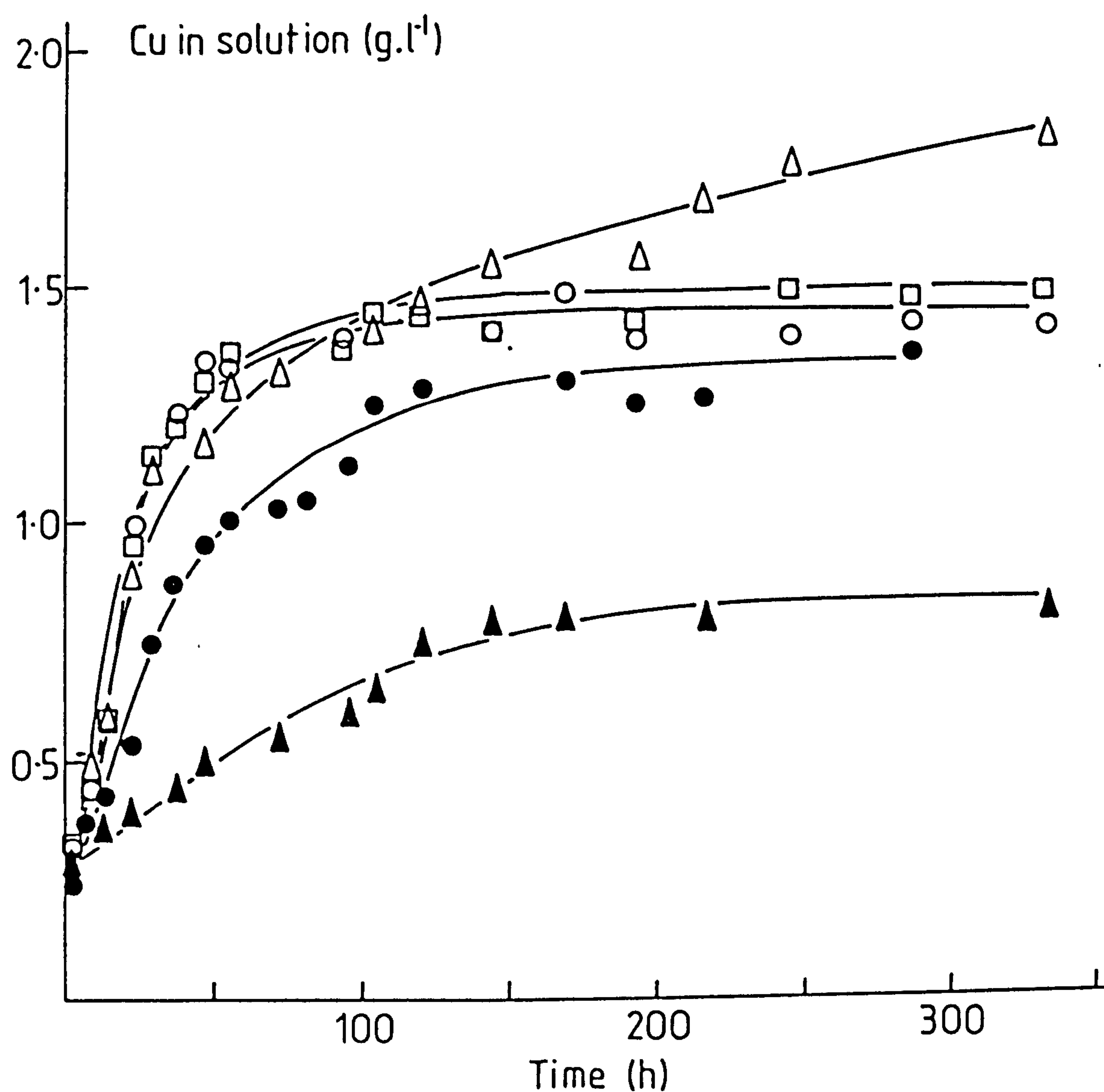


Fig. 58 Copper solubilization from chalcopyrite (10g.l^{-1}) during growth of the moderately thermophilic Birch Coppice isolate in the presence of yeast extract (0.2g.l^{-1}) at 38°C (●), 46°C (□), 50°C (○), 55°C (△) and 60°C (▲).

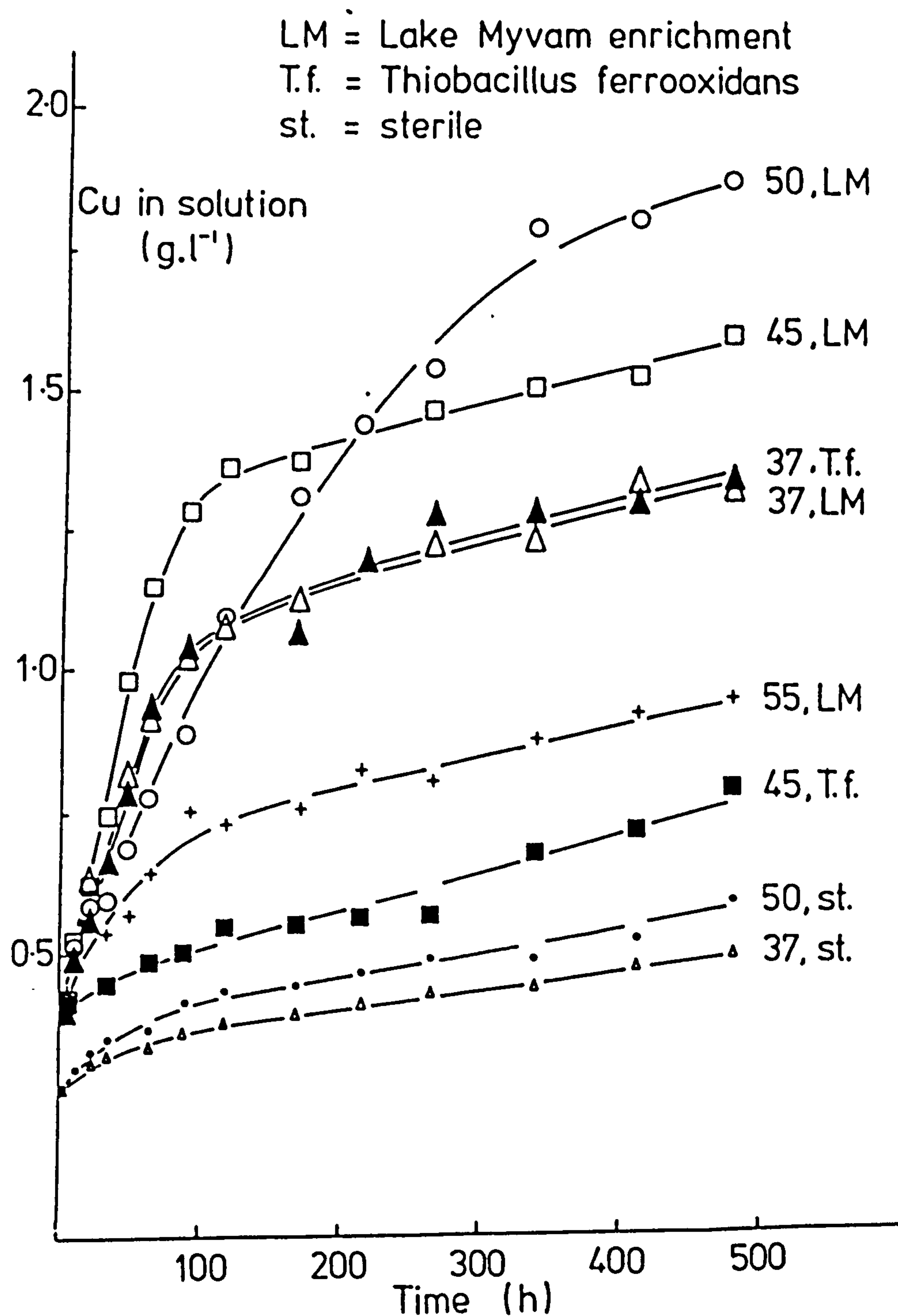


Fig. 59 Copper solubilization from chalcopyrite (10g.l⁻¹) during autotrophic growth of *T. ferrooxidans* (T.f.) and the Lake Myvam 40°-enrichment culture (LM) and in sterile controls (st.) at the temperatures (°C) indicated adjacent to the final solution copper values.

copper release to T. ferrooxidans at 37°C but at higher temperatures a higher copper yield was obtained until growth of the organism was inhibited at 55°C (Fig.59). Although the rate of copper release at 50°C was slower by this organism than at 45°C the final yield was higher. This rate inhibition at 50°C was not observed with either the Birch Coppice isolate (Fig.57) or the Alvecote isolate (Fig.60).

In contrast to pyrite oxidation by these organisms the final culture pH remained close (>1.8) to the initial pH.

7.4 EXTREMELY THERMOPHILIC BACTERIA: PYRITE OXIDATION

The results described in this section were obtained using 300 ml culture volumes in water-jacketed vessels with agitation provided by paddles driven by overhead motors (see Fig.1).

Growth of S. brierleyi could not be established on pyrite in the absence of yeast extract whether static or stirred cultures were used. In the presence of yeast extract (Fig.61) the rate of iron release became progressively faster until nearing complete mineral dissolution. This pattern of a gradually increasing rate did not change during serial sub-cultures and the data shown is taken from the fourth serial culture on pyrite.

In contrast, Sulfolobus (Lake Myvam), rapidly completed pyrite dissolution (10gl^{-1}) at its optimum temperature whether growing autotrophically or in the presence of yeast extract (Fig.61). Sulfolobus (Birch Coppice) showed a similar optimum temperature for pyrite oxidation of just below 70°C and a similar

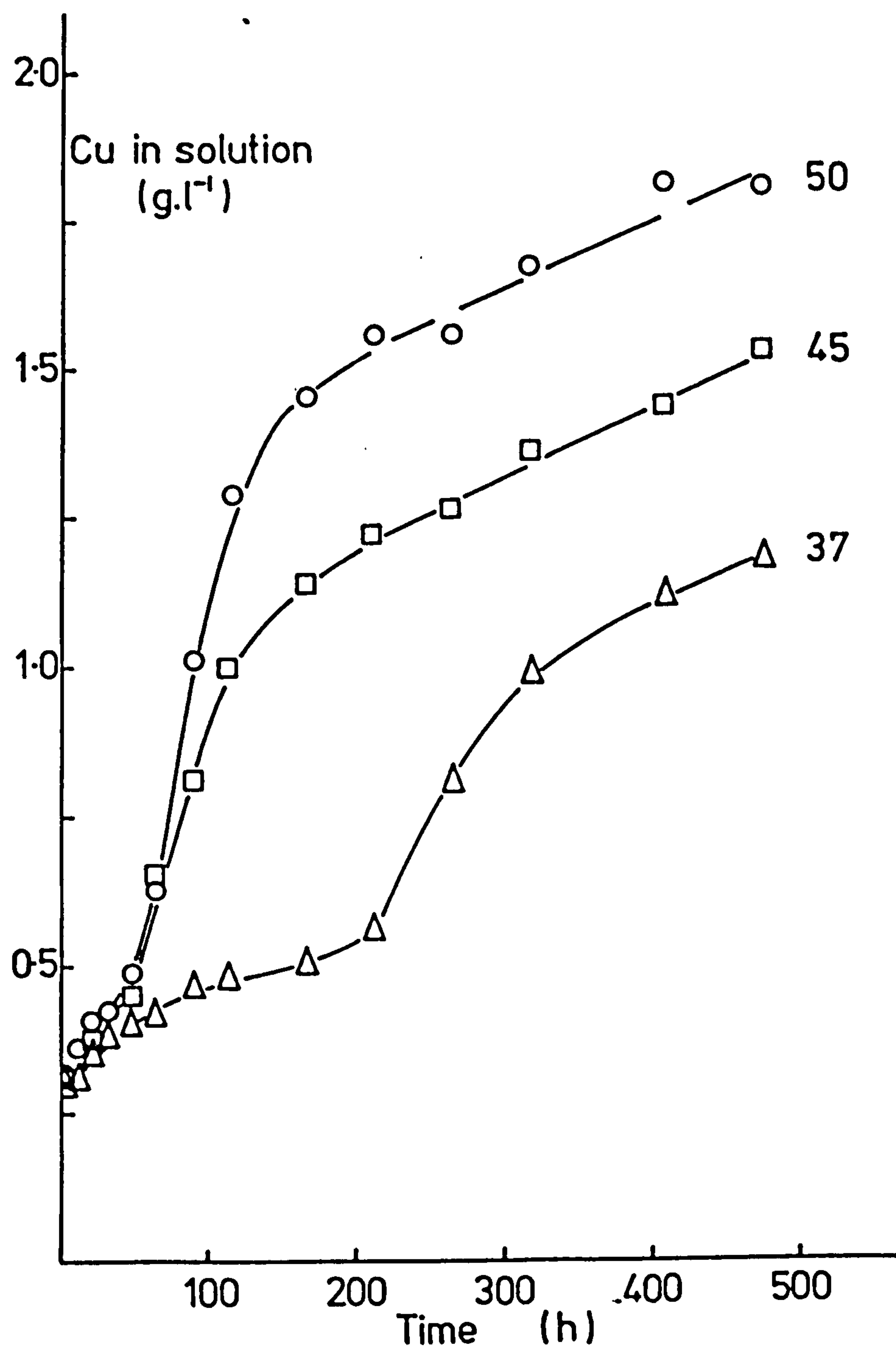


Fig. 60 Copper solubilization from chalcopyrite (10g.l⁻¹) during autotrophic growth of the Alvecote isolate at the temperatures (°C) indicated adjacent to the final solution copper values.

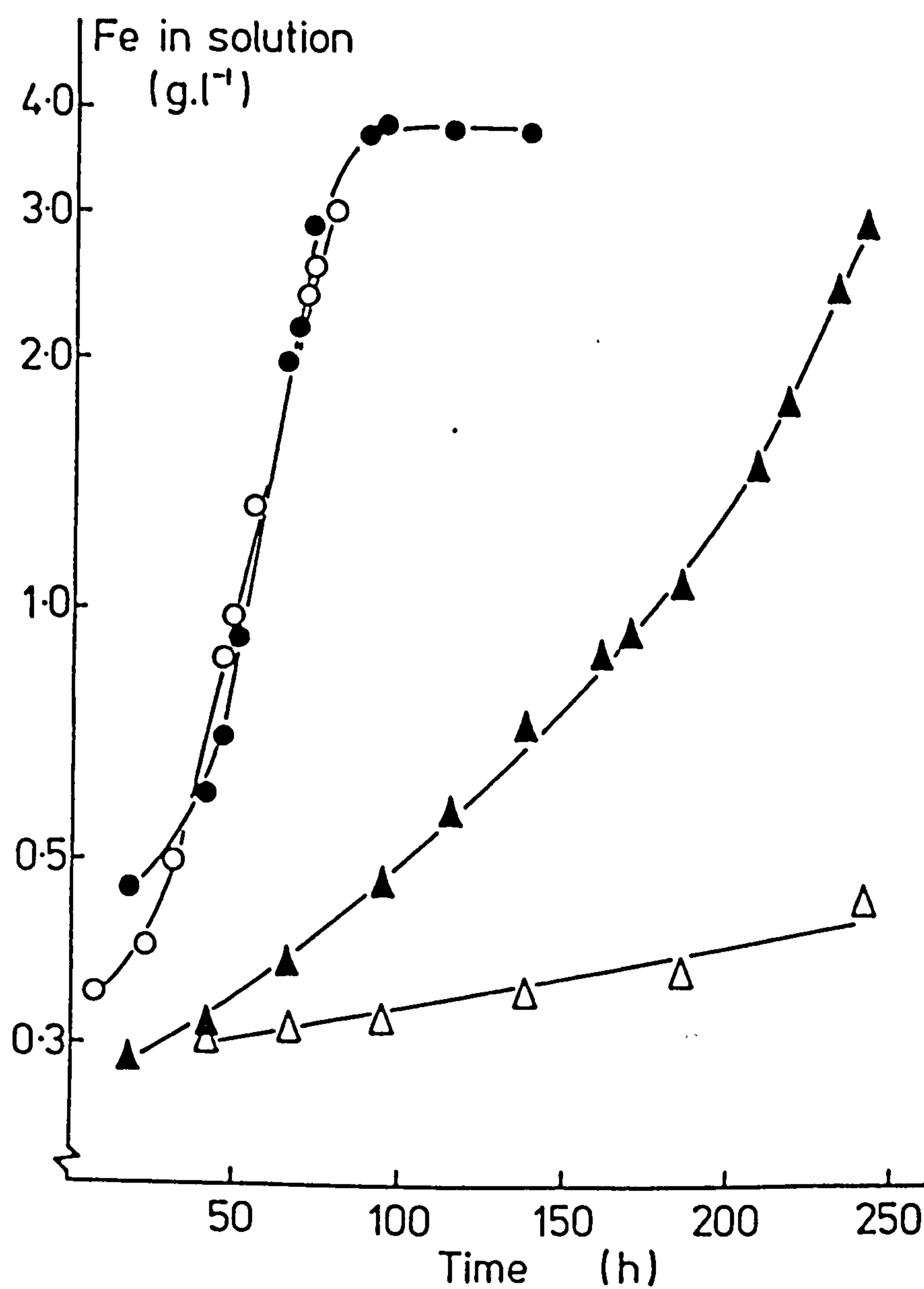


Fig. 61 The solubilization of iron from pyrite (10g.l^{-1}) at 68°C with (solid symbols) and without (open symbols) addition of yeast extract (0.2g.l^{-1}) by *Sulfolobus* (Lake Myvatn) (●, ○) and *Sulfolobus brierleyi* (▲, △).

rate of pyrite oxidation during autotrophic growth (Fig. 62) to Sulfolobus (Lake Myvam). Above the optimum temperature of the organism the rate of iron release was severely inhibited.

The growth of S. acidocaldarius 98-3, S. solfataricus and Sulfolobus B6-2 could not be established on pyrite whether in the presence or absence of yeast extract.

7.5 EXTREMELY THERMOPHILIC BACTERIA: CHALCOPYRITE OXIDATION

A comparison of Sulfolobus growth on chalcopryrite was made using an orbital shaking incubator and a shaking water bath in order to evaluate the effect of different mechanisms and hence the nature of the mineral agitation. This comparison was necessary because these experiments were performed before the stirred, water-jacketed vessels, noted in the previous section, were available and so water baths were required at temperatures above the operating limits of the orbital shakers. The copper solubilization rates and yields during growth of Sulfolobus (Lake Myvam) were extremely similar at 55°C (Fig.63) despite the different shaking systems. It was assumed therefore, that the method of agitation would not greatly have influenced the following results with chalcopryrite dissolution which were obtained using orbital shakers (at 60°C and below) and shaking water baths (at 65°C and above).

Growth-associated chalcopryrite oxidation by S. brierleyi was initially slow when compared with Sulfolobus (Lake Myvam). The yield of copper released during autotrophic growth of Sulfolobus (Lake Myvam) increased with temperature (Fig.64) up to

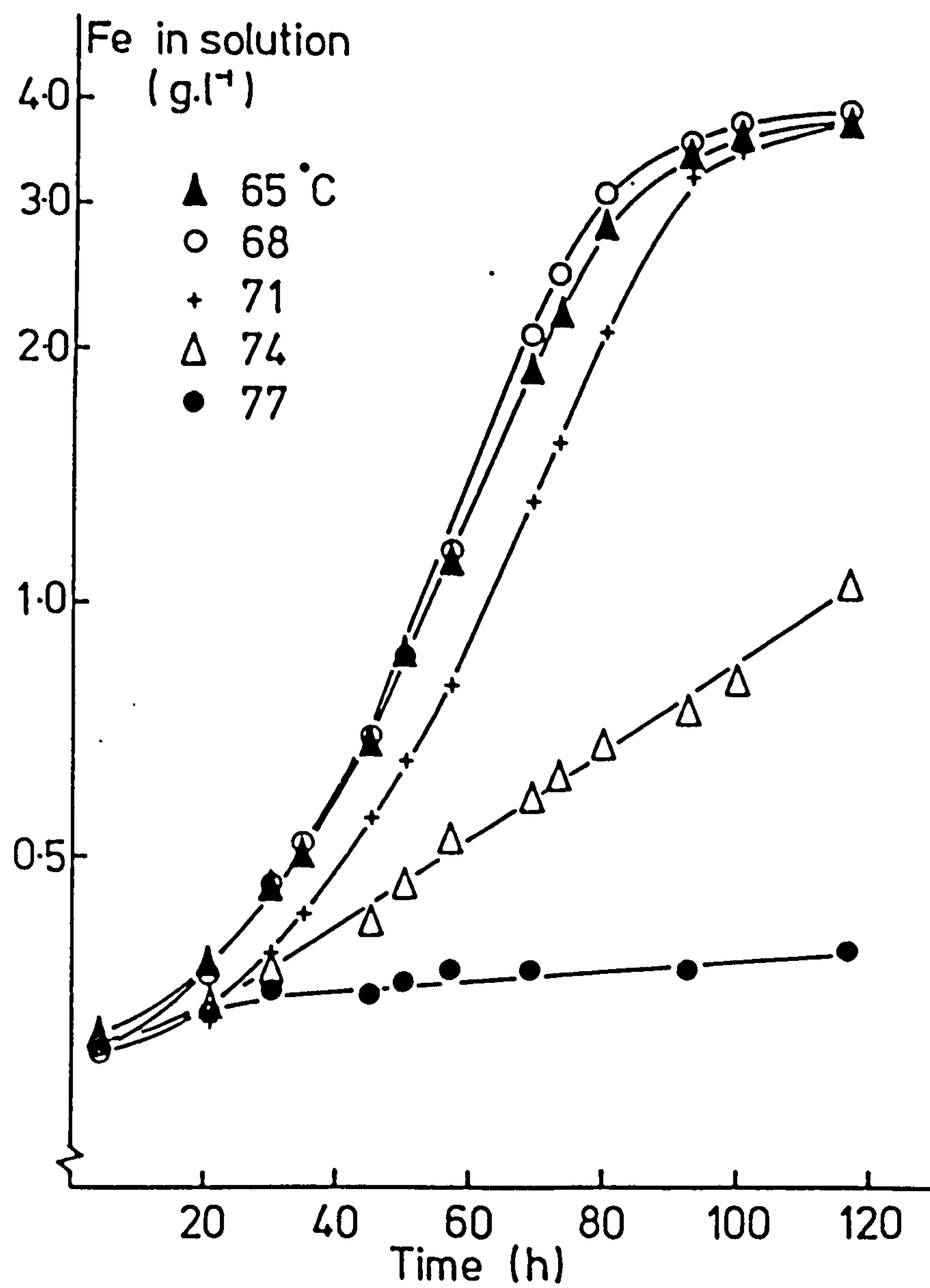


Fig. 62 The effect of temperature on the solubilization of iron from pyrite (10g.l⁻¹) during autotrophic growth of Sulfolobus (Birch Coppice).

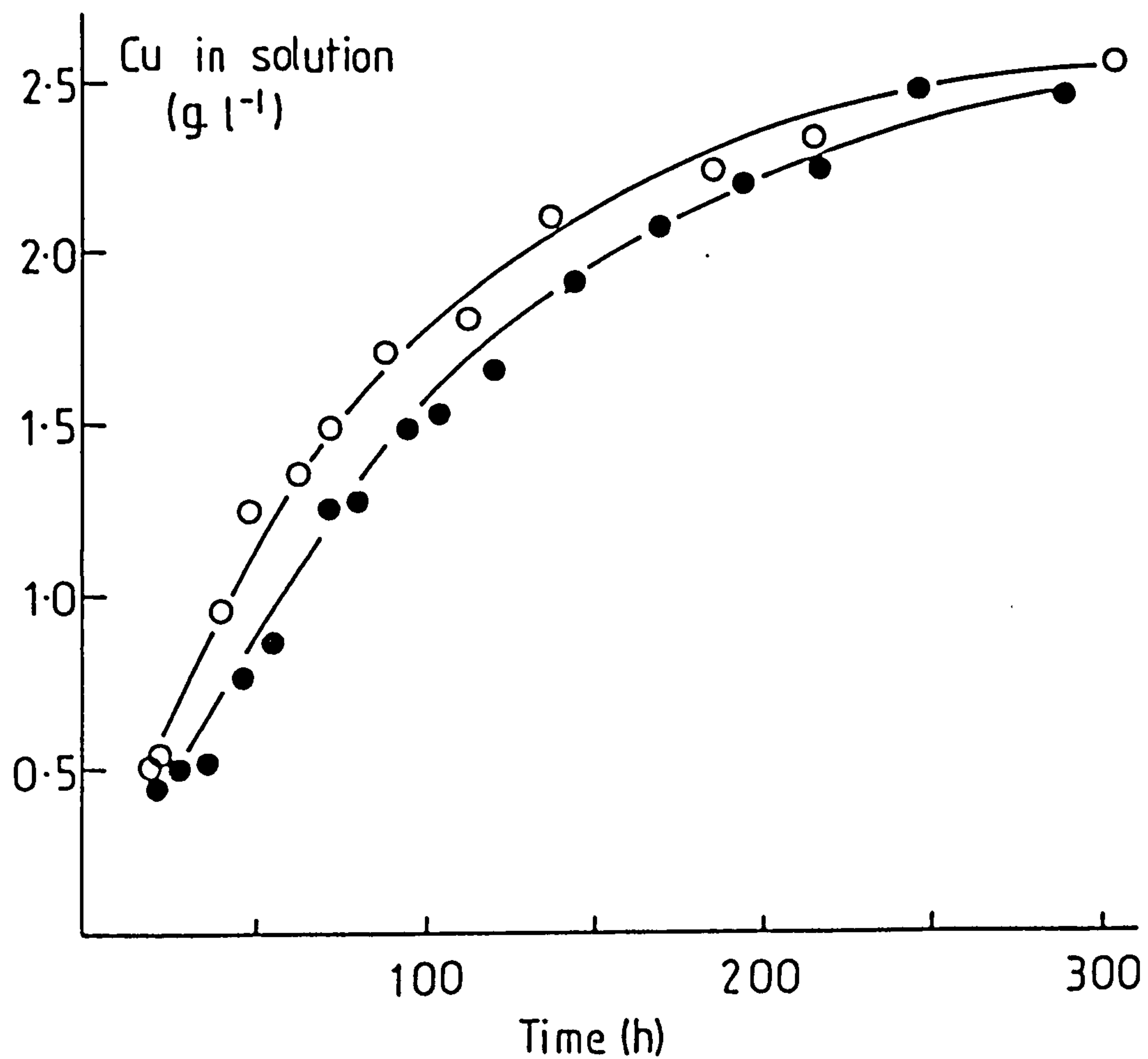


Fig. 63 Comparison of copper solubilization from chalcopyrite (10g.l^{-1}) by Sulfolobus (Lake Myvam) at 55°C with cultures shaken in an orbital shaker (●) and a water bath (○).

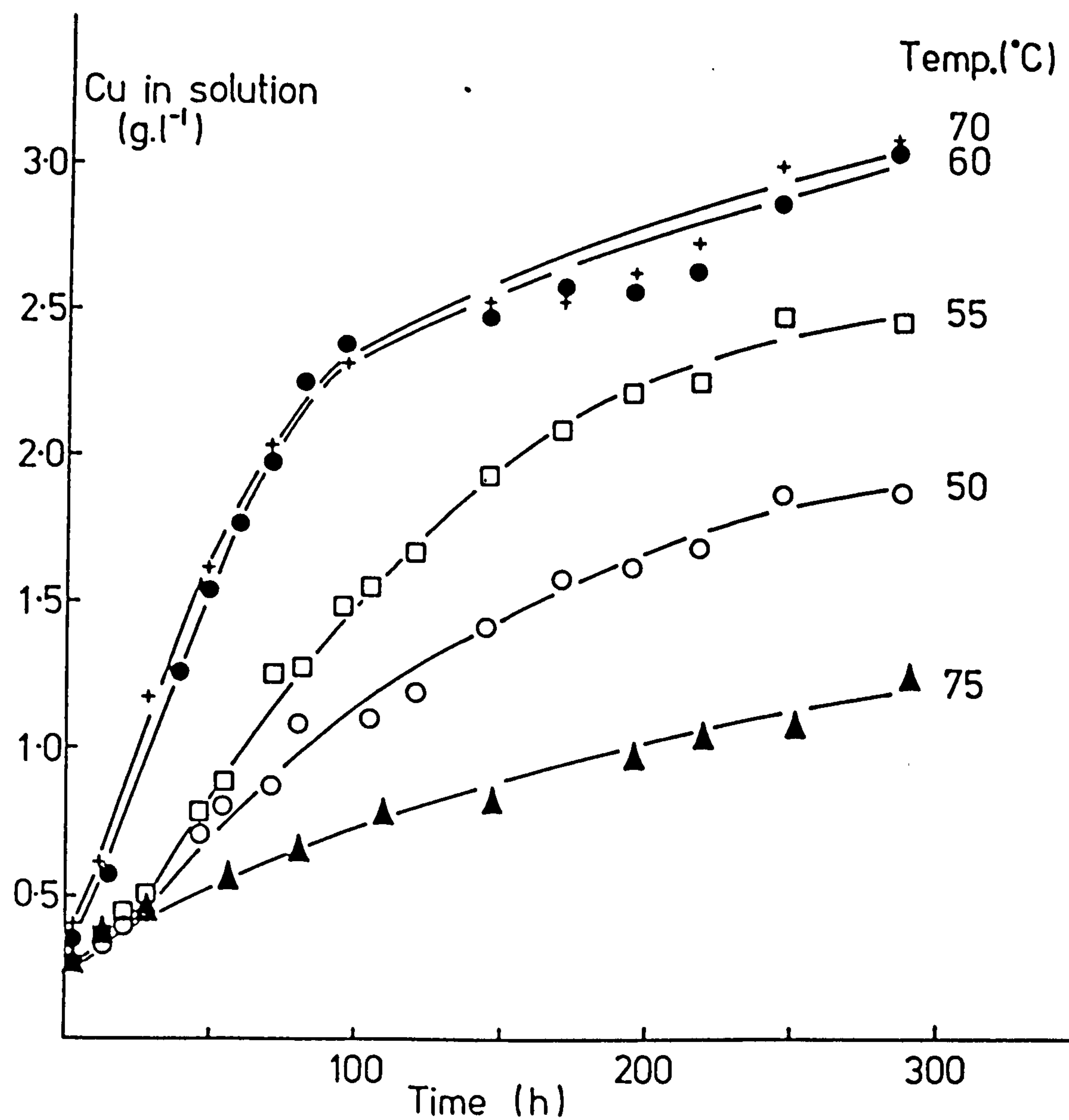


Fig. 64 The effect of temperature on copper solubilization from chalcopyrite (10g.l^{-1}) during autotrophic growth of Sulfolobus (Lake Myvam).

the optimum temperature of the organism when the yield decreased. At 70°C the chalcopryrite was leached to near completion and at 50°C the yield obtained was similar to that observed with the moderately thermophilic bacteria (Figs. 59 & 60). As observed during pyrite oxidation, the presence of yeast extract had little effect on the copper release from chalcopryrite (Fig.65).

The growth of S. acidocaldarius 98-3, S. solfataricus and Sulfolobus B6-2 could not be established on chalcopryrite.

7.6 DISCUSSION

When low levels of pyrite (10gl^{-1}) were used the yeast extract concentration limited the quantity of iron solubilized by thermophile TH1, as had previously been observed (Norris et al., 1980). Using only a defined organic carbon source, i.e. glutathione or glucose, the rate of iron solubilization was much slower than with yeast extract. The fact that the rate of iron solubilization was independent of the level of glucose also suggests that, if the glucose was being utilized to support chemolithoheterotrophic growth, its concentration was not rate limiting and that yeast extract was probably of more benefit to the bacteria through the provision of a wide range of organic growth supplements.

The demonstration of autotrophic growth of the moderate thermophiles precluded the further study of the effect of organic nutrients on mineral dissolution. It was clearly more important to pursue studies with cultures in the absence of organic supplements, as the presence of such supplements would be

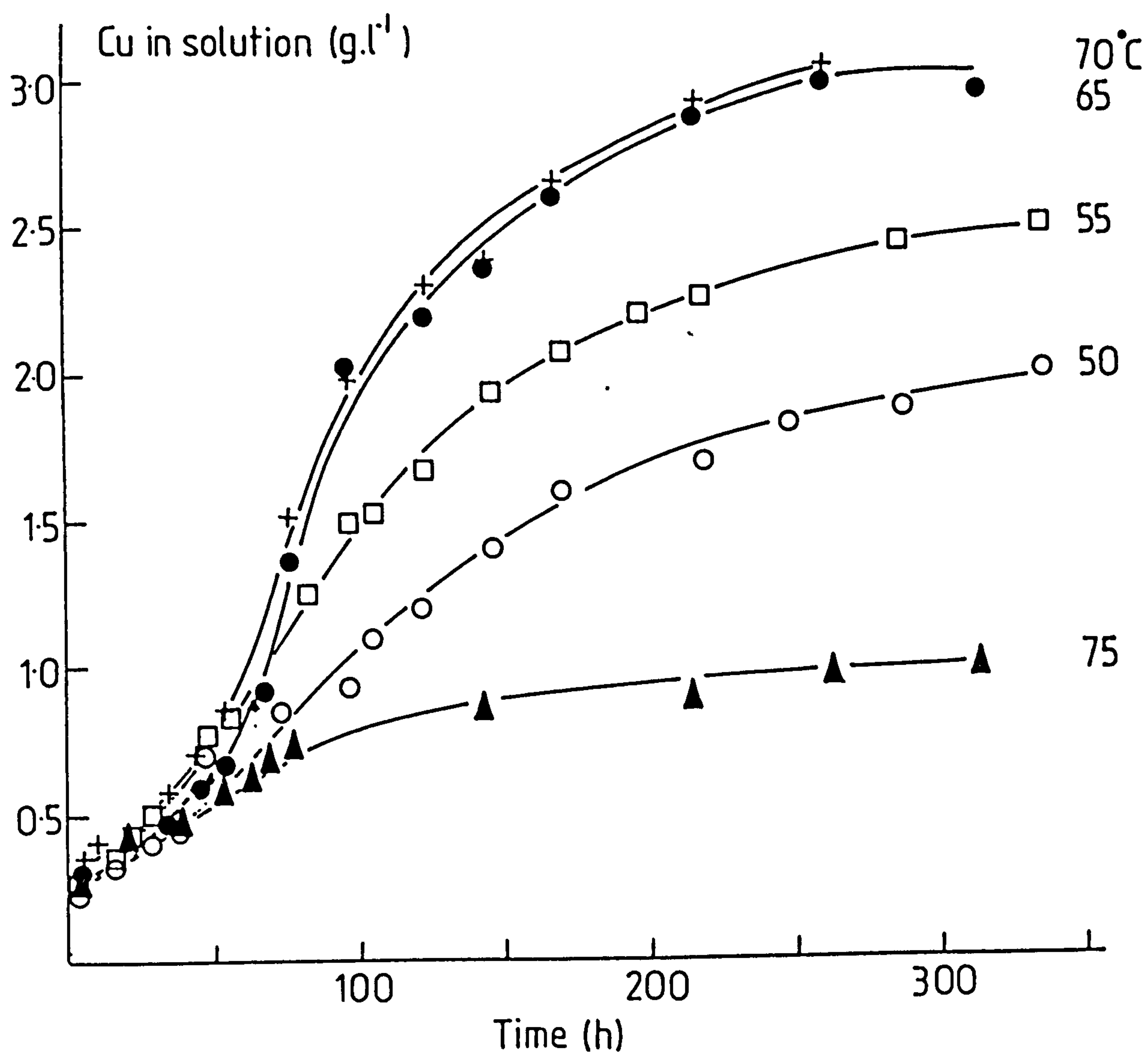


Fig. 65 The effect of temperature on copper solubilization from chalcopyrite (10g.l^{-1}) during growth of *Sulfolobus* (Lake Myvam) in medium supplemented with yeast extract (0.2g.l^{-1}).

undesirable for any consideration of the potential industrial application of such bacteria. The establishment of autotrophic pyrite oxidation by the moderately thermophilic bacteria demonstrated that they were able to fix carbon dioxide to enable substantial growth particularly if the levels were above that of atmospheric.

As with mesophilic cultures (Norris & Kelly, 1978; 1982), variations existed in the ability of enrichment cultures from different sites to oxidize pyrite. However, at pulp densities of 50g pyrite per litre a maximum of only 50% of the available iron was solubilized for all cultures at 50°C and increasing the concentration of yeast extract did not increase the yield. The resuspension procedure showed that the low pH was the limiting factor in continued pyrite breakdown. Alkali could not be added to maintain the pH at 2.0 due to iron precipitation (Atkins, 1978). A similar pH limitation has been demonstrated for T.ferrooxidans (Norris, 1983) but Leptospirillum ferrooxidans has shown a greater tolerance to acidic conditions.

The Birch Coppice moderate thermophile showed a lower optimum temperature for growth when growing autotrophically than in the presence of yeast extract. This reflects previous observations of variations in temperature ranges with growth conditions and substrates (Brierley & LeRoux, 1977). The Lake Myvam 40°C-enrichment culture showed a wide temperature range during which rapid pyrite oxidation occurred. This probably indicated the activity of T. ferrooxidans at 37°C-40°C and the

moderately thermophilic bacteria at the higher temperatures.

As with the oxidation of ferrous iron by Sulfolobus (Lake Myvam), there was little enhancement of pyrite oxidation in the presence of yeast extract. There was no adaptation by S.brierleyi to pyrite oxidation even in the presence of yeast extract. The observations that no growth could be obtained with S. acidocaldarius on pyrite are in conflict with reports of the use of this species for the desulphurization of coal (Kargi & Robinson, 1982a,b). Either mixed cultures or isolates with similar pyrite-oxidizing abilities to Sulfolobus (Lake Myvam) would appear to have been used in the desulphurization experiments.

The yield of copper from chalcopyrite increased with temperature for all cultures up to the highest temperature limit of the organisms. Although the Lake Myvam 40°C-enrichment culture showed a slower rate of copper solubilization at 50°C than 45°C the yield was still higher at 50°C. Sulfolobus (Lake Myvam) growth at high temperatures gave greater yields of copper than those obtained with the moderate thermophiles so that almost complete solubilization of copper occurred. Previously, chalcopyrite concentrations have often only been able to be leached to about 50% before regrinding was required to obtain further copper release (McElroy & Bruyensteyn, 1978) so the use of high temperature leaching using Sulfolobus species could provide an alternative method for obtaining high levels of copper extraction.

The final pH after growth on chalcopyrite by the moderately thermophilic and extremely thermophilic bacteria remained similar to the initial pH in contrast to the excess acid produced during pyrite oxidation. The pH may be influenced by a series of reactions which include the deposition of jarosites and basic ferric sulphates on the chalcopyrite surface (Lundgren & Silver, 1980) which could prevent further dissolution of the mineral. The rates of growth-associated ferrous iron and sulphur oxidation with Sulfolobus (Lake Myvam) were not greater than the rates shown with some moderate thermophiles in earlier sections of this work. Therefore, the more rapid and extensive chalcopyrite dissolution with the extreme thermophile would seem to result from chemical and physical factors influencing the deposition of the secondary minerals on the chalcopyrite surface, a deposition possibly prevented or less critical at the higher temperatures.

P A R T 8

CONCLUSIONS

Three major groups of bacteria were examined following the isolation of moderately and extremely thermophilic iron- and sulphur-oxidizing bacteria.

The moderately thermophilic iron-oxidizing bacteria which had been previously studied were isolated from hot spring and mineral sulphide mine areas. This study also included organisms from coal pile and coal spoil heap sites. The demonstration of autotrophic growth of such organisms from hot spring and from coal-rich sites was a significant step in establishing these organisms as worthy of further consideration for use in the field of biohydrometallurgy. A deliberate effort was not made to isolate a large number of different types of these bacteria but three groups were revealed and a fourth, strain TH3, had been described previously. It is therefore likely that many types of moderately thermophilic iron-oxidizing bacteria exist and a useful classification should follow further characterization which could include DNA-DNA homology studies to clarify any organism inter-relationships.

Although probably of less direct relevance than iron-oxidizing bacteria or the iron and sulphur oxidizing bacteria with reference to the extraction of metals, further study and classification of the moderately thermophilic sulphur-oxidizing bacteria is also required. The strains isolated and initially characterized in this study appeared similar to T. thiooxidans apart from their higher optimum growth temperatures but some diversity was indicated in the group through the cell protein electrophoresis comparisons.

The first examination of a Sulfolobus strain from a coal mine site was made in this study. Four groups of extremely thermophilic bacteria were recognized on the basis of

physiological and cell protein electrophoresis characteristics.

The observation of heterotrophic strains of Sulfolobus which appeared incapable of sulphur oxidation has emphasized the importance of developing a reliable technique for purifying cultures of extreme thermophiles before further study and classification of this group of organisms can be confidently undertaken.

The demonstration of rapid mineral degradation during the growth of both moderate and extreme thermophiles in the absence of organic nutrients has shown that these bacteria could be suitable for use in some industrial bacterial leaching systems, particularly any that might involve the agitation of mineral concentrates in reactor vessels. The observation that more extensive mineral solubilization, at least with low chalcopyrite concentrations, was obtained by leaching at high temperature in comparison with the range tolerated by the well-studied T. ferrooxidans has indicated a particular advantage of using thermophilic bacteria. Now that the capacity of some thermophilic bacteria for mineral sulphide dissolution has been described, the influences of factors such as high solution concentrations of toxic metals and high solid mineral concentrations on the activity of the bacteria need to be determined before their potential application in industrial processes can be fully assessed.

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